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From Genes to Traits: A Functional Genomic Study on the New World Screwworm Fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae)

De Genes à Fenótipos: Um Estudo de Genômica Funcional na Mosca-da-bicheira, *Cochliomyia hominivorax* (Diptera: Calliphoridae)

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of requirements for the degree of Doctor in Genetics and Molecular Biology, in the area of Animal Genetics and Evolution.

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Genética Animal e Evolução.

Orientador: Ana Maria Lima de Azeredo Espin
Co-orientador: Ana Carolina Martins Junqueira

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COMISSÃO EXAMINADORA

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Os membros da Comissão Examinadora acima assinaram a Ata de defesa, que se encontra no processo de vida acadêmica do aluno.

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa Genética e Biologia Molecular da Unidade Instituto de Biologia.

To my grandparents, Antônio and Maria, my parents, Fernando and Odalisia, my siblings, Natália and Guilherme, and my nephew to come, Vinícius.

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"*It seemed worthwhile therefore to examine more closely one example of a gene mutation affecting behavior and to ask two questions, (1) how does it bring about its effect? [and], (2) what part might it play in evolution?*"

- Margaret Bastock's on the *yellow* mutants of the fruit fly, *Drosophila melanogaster*. Her study was the first one to link a gene with behavior in insects, and probably any other animal.

(Bastock, 1956; *Evolution 10:421–439*)

R E S U M O

Apesar da sua natureza destrutiva, insetos considerados pestes nos proporcionam uma oportunidade impar de investigar como a evolução opera para a emergência de novos comportamentos e a conquista de nichos ecológicos. Em especial, identificar as bases genéticas subjacentes a sua biologia parece ser a chave para revelar as rotas evolutivas que levaram à suas adaptações ecológicas. A mosca-da-bicheira, *Cochliomyia hominivorax*, é um modelo promissor para estudar essas questões em califorídeos (Diptera: Calliphoridae). Enquanto espécies filogeneticamente próximas preferencialmente depositam seus ovos em matéria orgânica em decomposição, *C. hominivorax* infesta e alimenta-se de tecidos vivos de vertebrados de sangue quente. Surpreendentemente, e em oposição a sua importância, estudos acerca das bases moleculares envolvidas na escolha por hospedeiros vivos em *C. hominivorax* têm sido amplamente negligenciados. A fim de superar essa barreira, neste trabalho desenvolvemos protocolos eficientes de mutagêneses e estabelecimento de mutantes da mosca-da-bicheira utilizando a ferramenta de edição genômica CRISPR/Cas9 (Capítulo 1). Mutações direcionadas foram introduzidas no gene *yellow* de *C. hominivorax* (*ChY*), resultando no fenótipo *brown body* (*bwb*), o qual permitiu avaliar eventos mutacionais induzidos por Cas9. Elevadas taxas mutagênicas foram alcançadas ao entregar altas concentrações de complexos pré-formados de Cas9-sgRNA em embriões da mosca-da-bicheira. Mutações também foram introduzidas em *transformer* (*Chtra*), um gene chave para o desenvolvimento feminino em califorídeos. Mutações em *Chtra* resultaram na masculinização de fêmeas de *C. hominivorax*, sugerindo que este gene poderá se tornar um potencial alvo em futuras estratégias de manejo baseados em genética nesta espécie. Sinalização e percepção parecem evoluir em sincronia para moldar os comportamentos mediados por quimiorrecepção. Assim como em outros insetos, a mosca-da-bicheira utiliza-se de odores para encontrar hospedeiros. Neste contexto, o olfato deve ter tido um papel fundamental na mudança de preferencia por hospedeiros na linhagem de *C. hominivorax*. Para testar essa hipótese, nós examinamos o Receptor Co-receptor Olfativo Orco na mosca-da-bicheira (Capítulo 2). Insetos detectam odores em ambientes complexos através da expressão de Receptores Olfativos (ORs) e

Receptores Ionotrópicos (IRs). Orco é um co-receptor obrigatório para a função de todos ORs mas não para os IRs, permitindo com que comportamentos mediados pelo olfato possam ser estudados através da manipulação de um único gene. Orco em *C. hominivorax* (*ChomOrco*) é altamente conservado em Diptera, devido à forte pressões seletivas purificadoras. A expressão de *ChomOrco* é relacionada a diversos aspectos morfológicos e comportamentais da mosca-da-bicheira, sendo altamente representado nos apêndices olfativos da espécie. Uma linhagem mutante para *ChomOrco* foi gerada utilizando os métodos de CRISPR/Cas9 desenvolvidos anteriormente. Experimentos comportamentais revelaram que mutantes para *ChomOrco* não respondem aos odores associados aos comportamentos de busca por alimento e hospedeiros. Esses resultados sugerem que a via olfativa mediada pelos ORs está intimamente associada à escolha por hospedeiros, implicando que a evolução deve ter agido nestes genes para arquitetar a especificidade olfativa de *C. hominivorax*. Este estudo representa o início de uma investigação ainda maior que pretende desvendar as bases genéticas dessa extraordinária adaptação ecológica: De um organismo livre para um parasita obrigatório.

Palavras-chave: Genômica funcional; Genética reversa; Edição genômica; CRISPR/Cas9; Mosca-da-bicheira; Calliphoridae; Moscas varejeiras; Miíases; Ectoparasitismo; Parasitismo obrigatório; *brown body*; *yellow*; *transformer*; Orco; Olfato.

A B S T R A C T

Despite their destructive nature, insect pests provide us with an unparalleled opportunity to investigate how species evolve new behaviors and adapt to novel ecological niches. Especially, identifying the genetic bases underlying their lifestyle seems to be the key to unlock the evolutionary routes leading to their ecological adaptation. The New World Screwworm fly, *Cochliomyia hominivorax* (Coquerel, 1858), is a promising model for studying shifts in host-preference in blowflies (Diptera: Calliphoridae). While most closely related species lay their eggs on decaying organic matter, including carcasses, *C. hominivorax* oviposits on the dry borders of wounds and orifices of living warm-blooded vertebrates. Surprisingly, and in opposition to its associated economical and veterinary importance, the genetic basis of *C. hominivorax*'s preference towards living hosts has largely been neglected. To overcome this constraint, we developed highly efficient protocols for mutagenesis and generation of Screwworm knockouts (KOs) using CRISPR/Cas9 genome editing (Chapter 1). Site-directed mutations were introduced in the *C. hominivorax yellow* gene (*ChY*), resulting in the *brown body* phenotype (*bwb*), a visual trait to score for Cas9-induced mutations. High rates of somatic and germline mutagenesis were achieved when delivering Cas9-sgRNA pre-assembled complexes at high concentrations into Screwworm embryos. We next targeted *transformer* (*Chtra*), a major gene required for normal female development in blowflies. Experiments resulted in the masculinization of *C. hominivorax* females (intersex XX flies), suggesting that *Chtra* is a potential target for future control strategies in this species. Due to these outcomes, we anticipate that CRISPR technology will have a tremendous implication for the implementation of new genetic-based strategies to manage this important insect pest. However, and most importantly, the data described here provide the foundation for functional investigations on the evolution of natural traits in *C. hominivorax*. Signaling and reception have been shown to evolve in synchrony to shape olfactory landscape, thus chemosensory-mediated behaviors, in many dipterans. Like in other insects, the Screwworm relies on volatiles (odors) to find suitable hosts for oviposition. Therefore, we hypothesized that olfaction may have played a critical role in the shift of niche-preference in the *C. hominivorax* lineage. To test this

assumption, we functionally examined the Odorant Receptor Co-receptor gene (Orco) in Screwworm (Chapter 2). Insects detect odors through the expression of Odorant Receptors (ORs) and Ionotropic Receptors (IRs). Orco is an obligatory co-receptor for all ORs but not the IRs function, which in turn allows the screening of olfactory-mediated behaviors through the manipulation of a single gene. We found that *C. hominivorax* Orco (*ChomOrco*) is highly conserved within Diptera, as a consequence of a strong purifying selection regime. Expression of *ChomOrco* is linked to many morphological, developmental and behavioral aspects of the Screwworm, and is broadly represented in olfactory appendages of the adult flies. We next utilized this molecular information to knocking-out *ChomOrco* using the CRISPR/Cas9 methods previously described. Two-choice behavioral bioassays revealed that mutants for *ChomOrco* exhibit disrupted flight orientation, no longer responding to floral-like and host-associated volatiles. These results suggest that the OR-pathway is linked to host preference in Screwworm, and implies that evolution might have worked on the OR-pathway to architect the specific olfactory landscape in *C. hominivorax*. Overall, these results have several implications for functional investigations on blowflies, and will be a great addition to the current literature on Screwworm and other related blowflies. Yet, it is important to keep in mind that, the present study is just the beginning of a way longer investigation regarding the genetic basis of such extraordinary ecological adaptation: From a free-living to an obligatory parasitic fly.

Keywords: Functional genomics; Reverse genetics; Genome Editing; CRISPR/Cas9; New World Screwworm fly; Calliphoridae; Blowflies; Screwworm; Myiasis; Ectoparasitism; Obligatory Parasitism; brown body; yellow; transformer; Orco; Chemosensory Genes.

F I G U R E S

T A B L E S

G L O S S A R Y

Ablated "*antennaless*" flies = adults that had at least the 3rd antennae segment physically removed.

Biallelic mutations = Mutations occurring in both alleles of a *N* = 2 individual. Their kind can be the same or different.

Blowflies = Dipteran species bellowing to Calliphoridae family.

bp = Bases pair.

bwb = *Brown body* mutant phenotype.

C. bezziana = *Chrysomya bezziana*, the Old World Screwworm fly.

C. hominivorax = *Cochliomyia hominivorax*, the New World Screwworm Fly.

C. macellaria = *Cochliomyia macellaria*, the Secondary Screwworm Fly.

C. megacephala = *Chrysomya megacephala*, the Oriental Latrine Fly.

Cas9 = CRISPR-associated protein 9.

cDNA = Complementary DNA.

CDS = Coding DNA sequence.

Chtra = *transformer* gene from *C. hominivorax*.

ChY = *yellow* gene from *C. hominivorax.*

***ChYellow*$^{07/01}$** = *bwb*-mutant strain of *C. hominivorax* species.

***core*-Calliphoridae** = [[Lucilinae + Calliphorinae] + Chrysomyinae] (According to Rognes, 1997).

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats.

D. melanogaster = *Drosophila melanogaster*, the Vinegar fly.

D. suzukii = *Drosophila suzukii*, the Spotted-wing drosophila.

DSBs = Double-strand Breaks.

dsx = *doublesex* gene.

Ectoparasite = A parasite that lives on the outside of its host.

Episodic selection = "*Any sudden ecological disturbance likely to lead to a significant alteration in a species' population structure*" (Definition according to Brasier 1986).

Founders = Individuals that produced viable offspring for line establishment.

fru = fruitless gene.

F_x = Filial indicator.

GO05 = wt strain "*Goias 2005*" of *C. hominivorax* species, being kept at UNICAMP-BR.

G_x = Generation indicator after mutagenesis.

HDR = Homology-directed Repair pathway.

Heteroduplex DNA = double-stranded DNA originated through the recombination of single partially complementary strands.

Indel = Deletions and/or insertions.

IR = Ionotropic Receptor.

J06 = wt strain "*Jamaica 2006*" of *C. hominivorax* species, being kept at COPEG-PA.

Kb = Kilobases pair.

KI = Gene/Individual knockin.

KO = Gene/Individual knockout.

L. cuprina *= Lucilia cuprina*, the Australian Sheep Blowfly.

LA07 = wt strain "*Los Angeles 2007*" of *L. cuprina* species, being kept at NCSU-USA.

Lc1B/C = *bwb*-mutant strains of *L. cuprina* species.

Line = Genetic line or lineage; Related individuals carrying the same genetic trait.

Loss-of-function = Mutations that result in the less product or no function of a gene.

Ma = Million years ago.

Mosaic = Individuals hosting different kind of mutations in their cell populations.

mRNA = Messenger RNA.

Myiasis = "*The infestation of live humans and other vertebrates caused by dipterous larvae that feed on the host´s dead or living tissue, liquid body-substances, or ingested food*" (Definition according to Zumpt, 1965).

Necro-saprophagous = Species that feed on dead tissues and/or decaying organic matter.

NHEJ = Non-homologous End Joining repair pathway.

OR = Odorant Receptor.

Orco = Olfactory Receptor Co-receptor.

ORNs = Odorant receptor neurons.

OSNs = Olfactory sensory neurons.

PAM = Protospacer Adjacent Motif.

PCR = Polymerase Chain Reaction.

qPCR = Quantitative Real-time PCR.

RACE = Rapid Amplification of cDNA ends.

Reads = Fragments of DNA sequence obtained by sequencing.

RNAi = RNA interference.

RNPs = Ribonucleoprotein complexes.

RT-PCR = Reverse Transcription PCR.

Screwworms = Blowflies of the genus *Cochliomyia*.

Sensilla = Small hairs modified for perception of specific stimuli.

sgRNAs = Single-stranded guide RNAs.

Sham individuals = Individuals that goes through the experimental proceedures without actually performing the final treatment (Controls).

SIT = Sterile Insect Technique.

ssODN = Single-stranded DNA donor.

Stimuli = Sensorial signal.

Strain = Established population carrying the same genetic variant (e.g., a mutant strain).

T7EN1 = T7 endonuclease 1.

TM = Transmembrane domains.

tra = *transformer* gene.

UTR = Untranslated region.

VOCs = Volatile Organic Compounds.

wt = Wildtype individual.

ZsGreen = Plasmid pB[Lchsp83-ZsGreen], described by Concha et al. (2011).

ω = Normalized non-synonymous (d_n) to synonymous (d_s) substitution rate.

C O N T E N T S

INTRODUCTION TO THIS THESIS

The Beauty in a Beast

FLIES ARE AMAZING. The Order Diptera (two-winged flies or true flies) encompasses approximately 160,000 known species globally distributed (Pape et al. 2011). This implies that there are nearly 24 species of flies for each species of mammal on Earth (Burgin et al. 2018). Indeed, Diptera is among the "*big four*" megadiverse groups of insects, alongside with Coleoptera (beetles), Lepidoptera (moths and butterflies), and Hymenoptera (wasps, bees and ants), together accounting for the majority of animal life on Earth (Grimaldi and Angel 2005). The last estimations placed the origin of Diptera at 251 Ma, and it's diversification at the Jurassic-Cretaceous border ca. 150 Ma, concomitant with the spectacular radiation of flowering plants (Misof et al. 2014). However, the most fascinating explosion of speciation within Diptera occurred ca. 65 Ma, marked by the rising of the most diverse group of flies: The Schizophora clade (comprised of near 1/3 of all dipteran diversity). Low extinction rates are thought to be the primary cause of Schizophora diversification (Wiegmann et al. 2011), which occurred during the Cretaceous–Paleogene (K–Pg) mass extinction event (the one that extinguish all non-avian dinosaurs), although the association with Angiosperms, that dominated landscapes during that time, might have had a significant influence in their prevalence and speciation as well. Schizophora is classically divided into two large groups, named "Acalyptratae" and Calyptratae. While the evolutionary relationships among the former remain controversial, the monophyly of Calyptratae is strongly supported (Kutty et al. 2010; Wiegmann et al. 2011; Junqueira et al. 2016). The emergence of calyptrates represents the final period of radiation in Diptera, and includes species that are instantly recognized as "*true flies*", as for instance, the Tsetse fly *Glossina morsitans* (family Glossinidae), the house fly *Musca domestica* (Muscidae), the human

botfly *Dermatobia hominis* (Oestridae), and the oriental latrine blowfly *Chrysomya megacephala* (Calliphoridae).

Among calyptrates, the family Calliphoridae has received particular attention. Commonly known as blowflies[1], the members of this family are habitually found feeding and breeding on decaying organic matter, including decomposed cadavers (Guimarães and Papavero, 1999; Amendt et al., 2004). Indeed calliphorids are one of the first insects to arrive on corpses, highlighting their value in forensic entomology science (Erzinclioglu, 2003). Due to their direct interaction with microorganisms, blowflies are considerate major reservoirs and mechanical vectors for many pathogens (Junqueira et al. 2017), exposing their medical, veterinary and sanitary importance as well. Yet, although better known for these necro-saprophagous flies, the group spans an even great diversity of life-history strategies. The naturalist Erica McAlister, the senior curator at the UK Natural History Museum, has accomplished the hard task of classifying dipterans' life strategies into nine main behavioral groups, which were described in her former book entitled "*The secret life of flies*" (McAlister, 2017). Among them, The Parasites, which in her own words "*Their methods of survival are often disgusting but the evolutionary genius is admirable* (…) *the most intriguing and attractive of all flies*".

David Grimald and Michael Engel's meticulously reviewed the *Evolution of insects* (2005) states that "*Calyptratae flies have redefined the "art" of vertebrate parasitism, particularly the Oestroidea* (…)" [a superfamily that includes blowflies]. When any dipterous larvae developed in a living animal, such parasitism is known as myiasis[2] (Zumpt, 1965). There are about 80 known myiasis-causing blowflies, which typically feed on external host tissues; therefore they are classified as ectoparasites. Although many extraordinary forms of parasitism are found in the calliphorids (see Kutty et al. 2010; McDonagh and Stevens, 2011; Marinho et al. 2012, for some examples), none has challenged the scientific community for so long as the obligatory

[1] "A host or other substrates on which fly eggs have been laid is said to be *fly blown*. The use of *blow* in this context comes from the Old English term *blawan* and probably refers to the production of gas from bloated carrion containing maggots. This is also the source of the name *blowfly* for those flies that are most conspicuous at carrion, the Calliphoridae." (Scholl et al. 2019).

[2] "[Myiasis is defined as the] infestation of live humans and vertebrate animals with dipterous larvae which, at least for a certain period, feed on the host's dead or living tissue, liquid body-substances, or ingested food." (Zumpt, 1965).

ectoparasitism habit. The emergency and evolution of obligatory ectoparasitism lifestyle in blowflies has been the subject for scientific debate for over a century (Keilin, 1915; Zumpt, 1965; Erzinclioglu, 1989). We recently collected evidences showing that the speciation of blowflies occurred ca. 22 Ma, concomitant with the appearance of grasslands and the radiation of grazing animals (Junqueira et al. 2016; Appendix A). These results corroborated with the hypothesis held by the Brazilian zoologist Nelson Papavero (1977), that the emergence of early mammals in the Cretaceous had a significant impact on the diversification of the Oestroidea, as several lineages of this superfamily show an intrinsic relationship with them. Our analysis also supports previous molecular studies showing that the obligatory ectoparasitism habit evolved independently and multiple times during evolution of blowflies, derived from a saprophagous ancestral habit (Wallman et al. 2005; Stevens et al. 2006).

In a theoretical scenario, the diversification and spread of grazing mammals during the Oligocene-Miocene border created a favorable landscape to the radiation of calliphorids. This sudden speciation might have been followed by an intense inter- and intraspecific competition for ephemeral carrion resource. Some blowflies attracted by injured tissues of living animals might have been favored by this harsh condition. Primary and secondary facultative species (i.e., species that can be either free living or parasitic) are thought to have evolved from this intermediate stage. Some facultative lineages might have further developed the preference for living hosts, followed by the ability to colonize living tissues, and ultimately leading to the emergency of obligatory ectoparasitic species. It has been proposed that susceptibility to parasites due to artificial selection have provided new opportunities for free-living saphrophagous blowflies to infest live animals. Therefore, the facultative ectoparasitic habit in some lineages might have coevolved in a relative recent past alongside with animal domestication (Erzinclioglu, 1989). This seems to be the case for ectoparasitic blowflies of the genus *Lucilia* spp. (Stevens and Wall, 1997; Stevens et al. 2006). However, this artificial selection process seems to have had none effect on the evolution of obligatory ectoparasitic habit, which have evolved prior to human domestication (Stevens, 2003; Junqueira et al. 2016), althrought it might have had some effects on the geographic range and populational expations of Screwworm natural populations (Fresia et al. 2013).

While these pioneer phylogenetic studies shed some light into the evolutionary route by which the obligatory ectoparasitism lifestyle emerged in calliphorids, an inevitable question arose: What are the genetic bases that underlie such a remarkable shift in niche preference in blowflies? In this study, we decided to explore this question by adopting the New World Screwworm Fly, *Cochliomyia hominivorax* (Coquerel, 1858), as a model species to investigate the evolution of niche preference in obligatory ectoparasitic blowflies. Adult females of *C. hominivorax* oviposit on dry borders of orifices or wounds in living warm-blood vertebrates. After hatching, their larvae (maggots) infest and feed on the host's living flesh to complete development (Alexander, 2006). Although one might guess from its name - the "*Man Eater*", as roughly translated from the Latin – *C. hominivorax* is a parasite of a wide range of wild and domestic animals. This species is unable to develop in the absence of a living host, thus designating an obligatory ectoparasitic blowfly. "*Screwworm*" is the common name given to the blowflies of the Old World genus *Chrysomya* and the New World genus *Cochliomyia*. These two groups share a common last ancestor at the basis of the Chrysomyinae subfamily, spanning ca. 17 Ma of independent evolution (Junqueira et al. 2016). In addition to *C. hominivorax*, there is only another obligatory ectoparasitic screwworm in the world, the Old World Screwworm fly *Chrysomya bezziana* (Villeneuve, 1914). While the Americas consisted of the original territory of *C. hominivorax* (now restricted to South America and some parts of the Caribbean, further discussed), the natural range of *C. bezziana* spans through tropical and subtropical regions of Africa and Asia (Hall et al. 2001). The independent emergence of the obligatory ectoparasitic habit in these species offers us an unparalleled opportunity to investigate the genetic basis of adaptive traits in calliphorids, and how species evolve to occupy novel ecological niches. For clarity purposes, in the present study the terms "*screwworms*" and "*Screwworm"* will be used to refer to the *Cochliomyia* and *Chrysomya* genus and the *Cochliomyia hominivorax* species, respectively.

Classically, reverse genetic studies in calliphorids have been performed by interference RNA (RNAi)-mediated loss-of-function experiments (Concha and Scott 2009; Li et al. 2013). The RNAi technique relies on the destabilization and degradation of a target messenger RNA (mRNA). Therefore, modifications induced by RNAi are

transient, in other words, they are not heritable. While RNAi modifications can be directly used for reverse genetics, germline modifications are essential to establish strains, allowing deeper investigations on gene functions. Until now, the lack of efficient gene manipulation strategies has represented a barrier in dissecting the cellular and molecular basis that underlies the biology and physiology of the parasitic traits in Screwworm (and other blowflies). But you might have heard about CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats, and the CRISPR-associated protein 9; Doudna and Charpentier 2014). CRISPR, for short, is a relatively new genome editing technology that has revolutionized functional studies in insects, especially in the non-model ones (Bono et al. 2015; Mansourian et al. 2019). The CRISPR system is composed of two main components: A DNA endonuclease enzyme called Cas9 (i.e., an enzyme that cleaves DNA), and a small (17 to 20 bp) synthetic single-stranded guide-RNA (sgRNA). In laboratory conditions, these two components are capable of assembling ribonucleoprotein complexes (RNPs). When introduced into cells, these RNPs are guided to a specific genomic region by base pair complementary between the sgRNA and its targeted DNA sequence. Once the targeted genomic region is recognized, the sgRNA-guided Cas9 promotes double-strand breaks (DSBs) that will be preferentially repaired by the non-homologous end-joining (NHEJ) pathway. The NHEJ is an error-prone process that often results in the introduction of deletions and/or insertions (collectively called *indels*) around the Cas9 cut site. When the targeted genomic region is a protein-coding sequence, these mutations usually introduce premature stop codons or frameshifts that disrupt the protein function. In contrast to RNAi, modifications introduced by CRISPR are at the genome level, thus likely to be heritable, and allowing the development of mutant strains.

In Chapter 1, we report the first successful use of CRISPR technology in *C. hominivorax* (Paulo et al. 2019; Appendix B). The establishment of CRISPR methods in Screwworm represents a landmark for genome editing in calliphorids, serving as a guide for future functional studies in this diverse group of flies. In oposition to its significance for evolutionary studies, it's also important to notice that *C. hominivorax* is a major pest of livestock, causing severe losses for the industry, which are evaluated at 337 million US dollars per year in Brazil alone (Grisi et al., 2014). The considerable

economic impact caused by this pest fly encouraged the United States government to strike back using the Sterile Insect Technique (SIT; Knipling, 1985) to eradicate *C. hominivorax* from the continent. The program started during the late 1950s, resulting in the successful eradication of Screwworm from North America to the Panama-Colombia border, in 2006 (Scott et al. 2017). This unprecedented achievement in insect pest management is broadly cited in textbooks as the most successful example of the SIT technology, which has been used to manage Screwworm populations over the last 60-years. During the past few years, however, there is an engagement in developing new genetic-based strategies aiming major improvements on the effectiveness of the current SIT program (Concha et al. 2016), including novel, environmentally sustainable control technologies based on genome editing, such as CRISPR-based gene drivers (Scott et al. 2018). In this context, the first Chapter of this thesis was mostly dedicated to the biotechnological and applied perspectives of CRISPR on the management of Screwworm' natural populations. While the implementation of this technology on Screwworm's suppression programs seems to be far from now, the methods described in the first Chapter represent a significant improvement on the existing toolkit of molecular methods in Screwworm, creating the means by which the interface between, biology, evolution, ecology and genetics can be functionally examined.

As mentioned before, there are just a few studies regarding the evolution of the obligatory ectoparasitic lifestyle in calliphorids. Most hypotheses have been made based on time-scaled phylogenetic inferences correlated to actual niche occupancy by blowflies. Obligatory ectoparasitic lineages are likely to have evolved in sympatry with necro-saprophagous blowflies, in response to strong competition for resources. This condition may have favored species brave - *or desperate* - enough to lay their eggs into dead tissues of living hosts, thus giving the chance for opportunistic facultative blowflies to evolve. That finding comes from the fact that every obligatory parasitic lineage within Calliphoridae derived from a necro-saprophagous ancestral group, and most of the time having a facultative parasite as closely related species. For instance, the *C. hominivorax* species is the only obligatory parasite within the *Cochliomyia* genus, which forms a strongly supported monophyletic group with *Compsomyiops*, a group of carrion-feeder blowflies (Singh and Wells, 2011; Yusseff-Vanegas and

Agnarsson, 2016). All other *Cochliomyia* species are defined as carrion-feeders and (mostly secondary) facultative parasites. Therefore, the necrophagous habit is the most parsimonious ancestral state. From a facultative stage, it was just a matter of time before the ability to colonize dead tissues of living animals facilitated the adaptive radiation of an obligate parasitic lineage, while congeners remained active as carrion-feeders. There is currently insufficient reliable evidence to conclude exactly how the transition from dead to living tissues occurred, but the included-niche hypothesis is a model that possible fits (Schoener, 1974). Briefly, the model predicts that interspecific competition towards a shared source should force species to reduce niche overlapping to facilitate their coexistence. This can be achieved by niche shifts towards a less preferred resource, which increases specialization over time, ultimately leading to ecological divergences during speciation.

As in many other insects, *C. hominivorax* relies on olfactory clues to find hosts for oviposition (see review by Tomberlin et al. 2017 and within references). However, finding suitable hosts can be a hard task in a chemically complex environment. Therefore, it's expected that *C. hominivorax* olfactory morphology and molecular machinery to have evolved in synchrony with signals derived from its unique niche, creating a specific recognizable olfactory landscape (Hansson and Stensmyr, 2011; Syed, 2015). In this context, we hypothesized that olfaction may have played a critical role in the adaptive transition from a free-living to an obligatory ectoparasitic species within the Screwworm lineage. Here, in Chapter 2, we proposed to functionally examine this assumption by studying the evolution of olfactory perception in the Screwworm. In insects, two distinct highly divergent olfactory receptors families, named Ionotropic Receptors (IRs) and Odorant Receptors (ORs), are responsible for the acquisition of volatiles (odors) information (Carey and Carlson 2011). Interesting, the proper function of all ORs is dependent on a highly conserved Olfactory Receptor Co-receptor (Orco). Thus, it's expected that a failure to encode Orco would result in the silencing of the entire ORs family, but not the IRs. In this context, Orco loss-of-function provides a great system in which olfactory-mediated behaviors by the ORs and IRs pathways can be rapidly profiled (Mansourian et al. 2019). Hence, we conducted the genomic isolation, characterization, evolutionary and expression profiling of the Orco gene of the *C. hominivorax* species. (*ChomOrco*). We next used

our CRISPR targeted mutagenesis methods (Chapter 1, and Paulo et al. 2019) to interrogate *ChomOrco* for its consequences on Screwworm host-seeking behavior. The results suggested that changes in the OR-pathway are expected to have contributed significantly to niche shift and occupation by the *C. hominivorax* lineage. Although we have previously correlated gene expression with divergent larvae feeding preferences in screwworms (Paulo et al. 2017; Appendix C), the data described in Chapter 2 represents the first functional evidence of a gene linked to a behavior in the *C. hominivorax* species. We believe that, together with the implementation of the CRISPR genome editing methods, the data obtained during the investigations on *ChomOrco* will be a landmark for functional genomics in blowflies.

Sometimes it can be hard to look at a pest insect beyond its impact on human activities, but it's possible to find beauty in this duality. In the case of insects, a side effect of "*knowing your enemies*" is to appreciate them, their intriguing life history, fascinating behaviors (and the myths surrounding them), and the rise of evolutionary innovations within lineages. Genome editing methods has appear as a promising tool for new pest insect management strategies, but also offer us an unparalleled opportunity to better understand these little "*buzzing*" beasts, and consequently life on Earth. In the context of the *Cochliomyia hominivorax* evolution, these methods give us the chance to unlock one of the most remarkable ecological adaptations within blowflies: The shift from a free-living to an obligatory parasitic fly.

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CHAPTER 1

Specific Gene Disruption in *Cochliomyia hominivorax* Using CRISPR/Cas9 [3]

Abstract The *Cochliomyia hominivorax* species is considerated a major pest of livestock. Its larvae infest warm-blooded vertebrates and feed on host's living tissues, resulting in severe losses for the lifestock industry. As a serious pest, considerable effort has been made to develop genomic resources and functional tools aiming to improve its management. Here, we report a significant addition to the pool of genome manipulation tools through the establishment of efficient CRISPR/Cas9 methods for the generation of directed and inheritable modifications in the genome of this screwworm fly. Site-directed mutations were introduced in the *C. hominivorax yellow* gene (*ChY*) producing lightly pigmented adults, and confirming its orthology with *Drosophila melanogaster yellow* gene. High rates of somatic mosaicism were induced when embryos were injected with Cas9 ribonucleoprotein complexes (RNPs) pre-assembled with guide RNAs (sgRNAs) at high concentrations. Adult flies carrying disrupted *yellow* alleles lacked normal pigmentation (*brown body* phenotype) and efficiently transmitted the mutated alleles to the subsequent generation, allowing the rapid creation of homozygous strains for reverse genetics of candidate loci. We next used our established CRISPR protocol to disrupt the *C. hominivorax transformer* gene (*Chtra*). Surviving females carrying mutations in the *Chtra* locus developed mosaic phenotypes of transformed ovipositors with characteristics of male terminalia while exhibiting abnormal reproductive tissues. The CRISPR protocols described here are a significant improvement on the existing toolkit of molecular methods in calliphorids. Our results also suggest that Cas9-based systems targeting *Chtra* could be an effective means for controlling natural populations of this important pest.

Keywords: Functional genomics, reverse genetics, myiasis, New World Screwworm fly, gene drive, CRISPR/Cas9, *brown body, yellow*, *transformer*

[3] The analysis and results described in this Chapter were published as part of an original research artcle in the Journal G3 (2019) 9(9): 3045 – 3055 (See Appendix B for more details).

INTRODUCTION

The New World Screwworm Fly, *Cochliomyia hominivorax* (Coquerel 1858), is a major livestock pest and the only obligatory ectoparasitic blowfly in the Neotropical region (Alexander 2006). Adult females of this species are attracted to oviposit by odors emanated by a wide range of hosts, including man, domestical and wild animals (Zhu et al. 2017; Yan et al. 2018). After hatching, larvae (maggots) infest and feed on host's living flesh to complete development, which can ultimately lead to lethality if untreated. These infestations (known as myiasis) are responsible for severe economic impacts to the livestock industry, estimated in hundreds of millions spent annually in treatment, prevention and animal welfare (Alexander 2006; Grisi et al. 2014). Over the last 60-years, the Sterile Insect Technique (SIT) program has been successfully used to eradicate *C. hominivorax* from all North and Central America (see review by Scott et al. 2017). Currently, the mass rearing biosecurity facility, at the Panama-United States Commission for the Eradication and Prevention of Screwworm (COPEG), is responsible for the dispersal of millions of factory-grown radiated-sterile screwworm males and females in the Darien barrier zone, along the Panama-Colombian border. The main goal is to prevent re-introductions of endemic flies from South America, where infestations remain a serious problem.

More recently, species-specific genetic control strategies for population suppression and/or the introduction of novel harm-reducing genetic traits in wild populations became a promising alternative to the traditional pest control methods (Alphey and Bonsall 2018). These strategies also provide major improvements on the effectiveness of SIT, for instance, through the establishment of Transgenic-Sexing Strains (TSS), which carry a conditional female-specific lethal transgene into the species genome (Thomas et al. 2000; Heinrich and Scott 2000; Scott et al. 2014). As *C. hominivorax* is a high-consequence pest of livestock, considerable effort has been made to develop genomic resources and functional genomic tools (Concha and Scott 2009; Li et al. 2013, 2014; Anstead et al. 2015; Yan and Scott 2015; Concha et al. 2016). However, although Cas9 was recently used to create a single knock-in mutation in the *L. cuprina no blokes* (*nbl*) gene (Davis et al. 2018), routinely successful methods for

direct gene disruption or genome editing in calliphorids have not been developed, until now. The lack of an established tool capable of generating specific inheritable genomic modifications, that allow deeper investigations on gene functions, represents a barrier in dissecting the cellular and the molecular basis that underlies the biology and physiology of the parasitic traits of *C. hominivorax*.

To overcome this significant constraint, we developed highly efficient site-specific mutagenesis methods for the generation of *C. hominivorax* knockouts (KOs) using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9) genome editing technology (CRISPR/Cas9; Doudna and Charpentier 2014). Cas9 is a DNA endonuclease enzyme capable of assembling ribonucleoprotein complexes (RNPs) with small (˜20 bp) single-stranded guide RNAs (sgRNAs) in laboratory conditions. When introduced into cells, these RNPs are guided to a specific genomic region by base pair complementary between the sgRNA and its targeted DNA sequence, which is directly followed by a protospacer adjacent motif (PAM, usually NGG-3'). Once the target is recognized, the sgRNA-guided Cas9 binds to its sequence and promotes double-strand breaks (DSBs) that will be preferentially repaired by the non-homologous end-joining (NHEJ) pathway. As a consequence, this error-prone process commonly results in the introduction of deletions and/or insertions (collectively called indels) at the break site, leading to mutations which - *in protein-coding exons* - are typically premature stop codons or frameshifts that disrupt the peptide sequence and so the protein function (Carroll 2014). Due to its simplicity, efficiency and precision, CRISPR arises as a powerful genome editing technology, being successfully used to study gene function in vivo in a diversity of insects and other arthropods (see review by Sun et al. 2017). Typically, research projects target genes linked to evident phenotypes (Bassett et al. 2013; Li and Scott 2016; Khan et al. 2017; Li et al. 2017b, 2017a; Heinze et al. 2017; Meccariello et al. 2017; Perera et al. 2018; Choo et al. 2018; Aumann et al. 2018; Liu et al. 2018; Sim et al. 2019, to cite some examples), which allows a rapid and unambiguous screening for successful Cas9-mediated editing events. In addition to the current efforts to assemble and annotate the *C. hominivorax* genome (M.J.Scott, unpublished; A.C.M.Junqueira, unpublished), we believe that the establishment of the CRISPR technique will be a significant improvement on the existing toolkit of molecular methods in this species. Further, the

technology offers the opportunity to engage in studies on novel, environmentally sustainable pest control technologies based on genome editing.

METHODS

Fly rearing and mutant strain establishment

The wildtype (wt) strains J06 of *C. hominivorax* was used for this study. Screwworm flies were maintained in the USDA-ARS research site located within the COPEG biosecurity plant in Panama (life cycle showed in Figure S1), under conditions previously described by Concha et al. (2016). A *C. hominivorax* strain carrying biallelic mutations at the yellow locus (*ChY*) was obtained by randomly crossing single G_2 *brown body* (*bwb*; see results) males with J06 virgin females. The G_3 offspring were let to inbreed freely in cages and all the obtained flies at G_4 showing *bwb* phenotype were selected and interbred to establish the heteroallelic mutant strain *ChYellow*$^{07/01}$ (see crossing scheme in Figure S2).

sgRNAs and Cas9 protein

Single guide RNAs (sgRNAs) were designed by examining both DNA strands in the exons of each investigated gene for the presence of protospacer-adjacent motifs (PAM) with the sequence NGG-3' (where N is any nucleotide) using the standalone version of CRISPOR tool (Concordet and Haeussler 2018). Considered sgRNAs sequences were 17-20 bp in length (excluding PAM) with in silico predicted minimal off-target effects on the genomes. Scanned sgRNAs containing GG before the PAM sequence (NGGNGG-3'), which may improve sgRNA efficacy (Farboud and Meyer 2015), and one or two 5'-end G at the start of the target sequence, which facilitates transcription by T7 RNA polymerase (Bassett and Liu 2014), were selected when possible. Replacement and additions of 5'-end guanines to the guide sequences were made when necessary. Synthesis of sgRNAs was performed as described for *Drosophila melanogaster* (Bassett and Liu 2014) with minor modifications. *C. hominivorax* templates were generated by step-up PCR in a 100 µl final volume containing 1x Phusion HF buffer, 200 µM of dNTPs, 2 U of Phusion DNA polymerase (NEB) and 0.5 µM of each CRISPR sgR-Specific-T7-FWD and sgR-Universal-REV primers. Amplification

conditions included an initial denaturation step of 98° for 2 min, followed by 10 cycles of 98° for 10 sec, 60° for 30 sec and 72° for 15 sec. Annealing temperature was increased to 65° for the subsequent 25 cycles of amplification, followed by a final extension at 72° for 10 min. Expected 100 bp amplicons were confirmed by electrophoresis in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) 2% agarose gels stained with GelRed, purified using the QIAquick PCR Purification Kit (Qiagen) and quantified with a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific). In vitro transcription of the sgRNAs was carried out using the MEGAshortscript T7 Transcription Kit (Ambion) and 300 ng of DNA template for a final volume of 20 µl. Reactions were incubated at 37° for 4 h followed by TURBO DNase (Ambion) treatment using 2 U for a further 15 min at 37°. Transcriptions were extracted with Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v, pH 6.7), precipitated with isopropanol and left overnight at -20°. RNA was collected by centrifugation, washed once with 85% ethanol and resuspended with RNAse-free ddH_2O. Concentrations of the sgRNAs were measured using Nanodrop, adjusted to 2 µg/µl, aliquoted and stored at -80° until use. Cas9 mRNA was in vitro transcribed using the mMessage mMachine T7 Kit and polyadenylated using the Tailing Kit (Ambion). Recombinant Cas9 protein was obtained commercially (PNA Bio or NEB EnGen Cas9 NLS, S. pyogenes), diluted to 1 µg/µl in DNAse-free ddH2O, aliquoted and stored at -80° until use. Designed sgRNA can be found in the Supplemental Material, Table S1.

Microinjections

Ribonucleoprotein complexes (RNPs) were pre-assembled by incubating Cas9 protein (500 ng/µl or 360 ng/µl) with sgRNA (200 ng/µl or 145 ng/µl) in a Sodium Phosphate Buffer (supplied with 300 mM of KCl to prevent Cas9 aggregation; Burger et al. 2016) at 37° for 10 - 30 min. For dual-targeting experiments, RNPs were pre-assembled using each sgRNA separately and then 1:1 mixed prior to the injections. To prevent needle clogging, the injection cocktail was centrifuged at top speed for 10 min at 4°C and the upper portion spun through a 0.45 µm filter column (Millipore) and collected in a clean tube which was maintained on ice during the experiments. Needles were prepared in a P-2000 needle puller (Sutter Instrument) using quartz capillaries with filament (O.D.: 1.0 mm I.D.: 0.7, 10 cm length), and opened during injections by contact with the

chorion of an embryo or beveled using a BV-10 Micropipette Beveler (Sutter Instrument). Adult *C. hominivorax* 6 d-old females were stimulated to lay eggs, which were then dissociated with KOH 4% (w/v) for 2 min and gently rinsed in ddH_2O. Treated embryos were aligned on a double slide tape in concave well microscopy slides, dehydrated in a silicagel chamber for 3 - 10 min and then covered with Halocarbon 27 oil (Sigma). Cocktails were injected though the chorion membrane into the posterior end of pre-blastoderm embryos (less than 40 min old; Figure S1). Injections were performed using a XenoWorks Micromanipulator connected to a Digital Microinjector (Sutter Instrument) device set for a "*continuous*" injection mode. After injecting *C. hominivorax* embryos, excessive oil was carefully removed and injected embryos were rehydrated by ddH_2O washing. Slides were placed in a petri dish containing humidified paper towel, and a small amount of larvae diet was placed surrounding the embryos. Plates were placed into a hyperbaric oxygen chamber and incubated overnight at 37° with 80% relative humidity. Eclosing first instar G_0 larvae were collected under white light using a Leica M165FC stereomicroscope, placed on diet and reared until adulthood, when mosaic adults were visually screened for the *bwb* mutation.

Genotyping

Genomic DNA was extracted using the DNAreleasy Advance Direct Lysis Kit (Bulldog bio) or DNAzol reagent (Invitrogen). PCR reactions were carried out for a final volume of 50 µl containing 1x Phusion HF buffer, 80 µM of dNTPs, 0.4 U of Phusion DNA polymerase (NEB) and 0.1 µM of each target specific forward and reverse primers. Samples were cycled under the following conditions: 98° for 2 min, 35 cycles of 98° for 10 sec, 60° for 30 sec and 72° for 30 sec, followed by a final extention step at 72° for 5 min. PCR products were verified by electrophoresis in a 1.5% agarose gel and purified as described above. Blunt-ended fragments were A-tailed with 1x PCR buffer, 200 µM of dATP, 5 U of Taq DNA Polymerase (QIAGEN) and 6 µl of previously purified PCR product. Reactions were incubated at 70° for 30 min and directly TA cloned into a pGEM-T Easy Vector (Promega). Plasmids were extracted using an alkaline lysis protocol and then digested with 20 U of EcoRI (NEB) at 37° for 2 h. Cloned fragments were verified by electrophoresis and submitted to Sanger sequencing in an ABI 3730xl

DNA Analyzer (Applied Biosystems), using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the universal primers M13-Forward and M13-Reverse. Raw reads were analyzed using FinchTV application (Geospiza Inc.) and assembled with CAP3 (Huang and Madan 1999). Contigs were mapped against the reference sequence (wildtype) using the BWA-MEM algorithm (Li 2013; relaxed parameters used: -A 4 -O 2 -L 5 -B 4 -E 1 -U 10), and analyzed by CrispRVariants package v.1.8.0 (Lindsay et al. 2016). T7EN1 cleavage assays were performed by denaturing 10 µl of unpurified PCR product at 95° for 10 min in NEBBuffer #2 (NEB). Reactions were then incubated at room temperature for at least 1 h for amplicon hybridization. Heteroduplex DNA were digested with 2 U of T7 endonuclease I (NEB) at 37° for 20 min and the entire reaction volume resolved in a 3% agarose gel electrophoresis in cold 1x TBE buffer. Genotyping primers specifications can be found in Table S2.

Data availability

The *C. hominivorax yellow* gene (*ChY*) was annotated from the current genome draft assembly (M.J.Scott, unpublished) and it is available upon request, while *tra* gene (*Chtra*) was retrieved from GenBank (accession number JX315618). All additional information is available as Supplementary Material at the end of this chapter.

RESULTS

Cas9-induced somatic and inheritable *brown body* mutation (*bwb*) in the *yellow* locus of *Cochliomyia hominivorax* (*ChY*)

Aiming to demonstrate the potential of gene specific knockout in the screwworm fly using the CRISPR/Cas9 technology, we selected *yellow* (*ChY*) as our candidate target gene. The *ChY* displays a high sequence and structure conservation with the *yellow* gene of *Drosophila melanogaster* and other dipterans (Figure S3), which generally implies a functional conservation as well. Thus, we hypothesized that *ChY* loss-of-function would result in the non-lethal *brown body* (*bwb*) mutant phenotype, as described for the housefly, *Musca domestica* (Heinze et al. 2017). Fully–developed *bwb* adults are expected to lack normal melanization, showing a noticeable brownish cuticle colored body, which in turn would be a simple phenotype to detect and score

successful Cas9-mediated mutagenesis in *C. hominivorax*. In this context, two sgRNAs targeting the second exon of the *ChY* (Figure 1A) were separately mixed with a high concentration of Cas9 purified protein (500 ng/µl) to form ribonucleoprotein complexes (RNPs). Pre-assembled RNPs were subsequently mixed together and co-injected into 280 early syncytial screwworm embryos. In total, 38 adults were obtained (surviving rate: 13.6%), of which 26 showed lighter pigmented cuticle in a mosaic manner (9 ♂ and 17 ♀; mutagenesis rate: 68.4%; Figure 1B, second column). These outcomes genetically confirmed that the *ChY* represents the *yellow* ortholog in *C. hominivorax*, and that disruptions in this gene promote the appearance of the *bwb* phenotype. Additional experiments are underway to evaluate if similar mating behavior defects found in yellow mutants of *D. melanogaster* (Bastock, 1956) are also observed in *C. hominivorax*. Since the *bwb*-mutation is recessive and autosomal in *C. hominivorax* (see results below) only mutations in both copies of *ChY* will result in the lighter pigmented body phenotype. Therefore, the results also revealed that the CRISPR/Cas9 protocol developed here is capable of inducing somatic biallelic lesions in a large number of individuals, with the vast majority of the mutated cells visible in the posterior (abdomen) and some at the anterior tissues (thorax and legs) of the G_0 flies (Figure 1B).

While somatic mutants can be directly used for reverse genetic studies, germline gene editing is essential to establish stable mutant lines. To evaluate the inheritance capability of the Cas9-induced mutations in the *ChY* gene, nine G_0 mosaics were individually inbred (each crossing consisted of 1 *bwb* G0 ♂ X 1 - 2 *bwb* G0 ♀). Out of the nine putative founder crossings, only two resulted in viable eggs, indicating that the microinjection process might have caused some damage to the precursors of the reproductive tissues during the development of the embryos. Additionally, all the 194 screened G_1 flies were phenotypically wildtype (wt), suggesting that these individuals were monoallelic mutants for *bwb*.

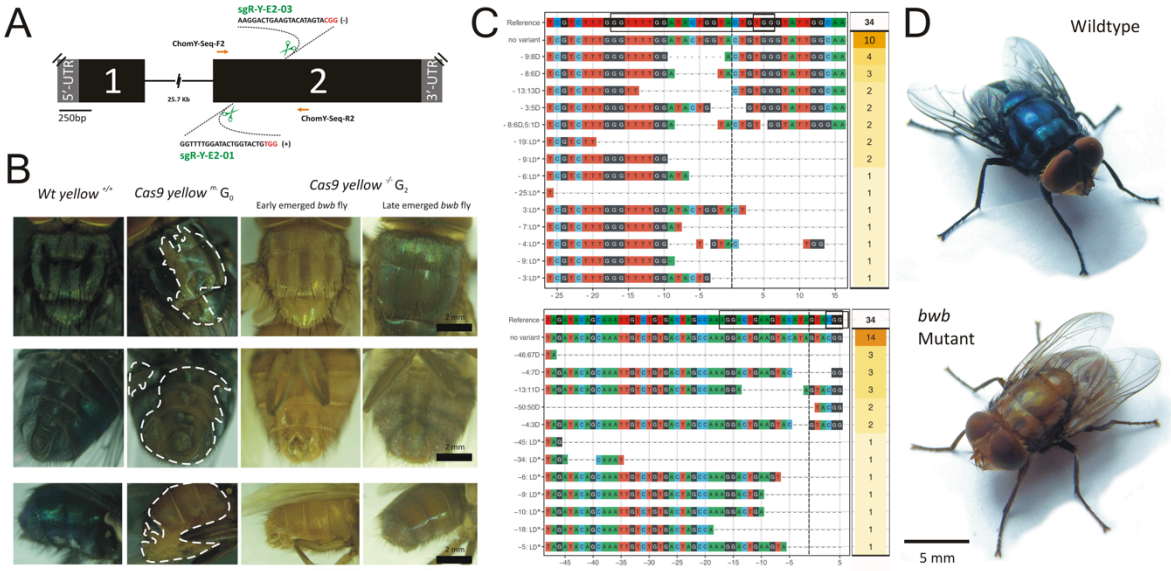

Figure 1. Cas9-mediated disruption of the *yellow* loci (*ChY*) promotes the *brown body* mutation (*bwb*) in the screwworm fly, *Cochliomyia hominivorax*. **(A)** Schematic of *ChY* genomic organization with exons as numbered black boxes, introns as lines and untranslated regions (UTRs) as gray boxes. Two sgRNAs were designed 368 bp apart to dual-target *ChY* exon 2. Cas9 RNPs targeted sites are indicated by scissors with their respectively sgRNA (PAM motifs are shown in red). Genotyping primers are indicated as orange arrows. **(B)** The *bwb*-mutant flies (leftmost) in comparison with the wildtype (rightmost) phenotype. The presence of yellowish body areas in the G_0 flies reveals biallelic hits in the *ChY* loci (regions encircled with a white broken line). Knockout individuals were obtained at G_2. The *bwb*-mutant flies showed a partial recover of the cuticle pigmentation some hours after the emergence. **(C)** Sequencing confirmation of target-specific disruption of the *ChY* loci at sgRNAs target sites. Alleles recovered from G_0 founders were mapped against the wildtype reference sequence and analyzed using the CrispRVariants pipeline (Lindsay et al. 2016). Targeted sites and PAM motifs are boxed. Predicted Cas9 cut sites are indicated by vertical black dashed line. LD* indicates alleles with large deletions between both sgRNAs (see Figure S4 for a more comprehensive alignment). **(D)** Habitus (i.e., general appearance) comparison between flies from the wildtype strain J06 (above, greenish-blue colored) and early emerged *bwb*-mutant strain *ChYellow*$^{07/01}$ (bellow, metallic yellowish).

In order to obtain *ChY* knockout strains (e.g., flies carrying *bwb*-mutations in both copies of the gene) all obtained G_1 flies were allowed to interbreed freely in cages (massive sibling crossing). The *bwb* mutant phenotype was observed in newly emerged G_2 individuals, revealing that Cas9-induced mutations are inherited in screwworm. The newly emerged *bwb* mutants were visually identified by the presence of a fully metallic yellowish colored cuticle (Figure 1B, third column), which becomes brownish some hours after the emergence (Figure 1B, fourth column), presumable due to the activity of other genes in the pigmentation pathway (Massey and Wittkopp 2016). A group of 52 flies (29 ♂ and 23 ♀) was selected to start a mutant strain colony in the laboratory.

Total DNA extractions were performed for the G_0 flies that produced viable eggs after crossings. We confirmed that the *bwb* mosaicism observed in these

founders (e.g., flies that produced viable eggs) was caused by the loss-of-function mutations in the *yellow* gene, by performing a T7 endonuclease 1 assay (T7EN1), which revealed the presence of indels at the specific Cas9 targeted sites within *ChY* (Figure S4). Subsequently, the same PCR amplicons were pooled, TA-cloned and Sanger sequenced (*n* = 34 clones) in order to sample the allele variants introduced by Cas9 mutagenesis. A diversity of mutated allele sequences was recovered (Figure 1C and S5), including variants exhibiting small (spanning from Δ -3 to -13 bp), medium (Δ -50 to -67 bp), and a considerable number of large deletions between both sgRNAs (Δ -360 to -423 bp in 29.4% of the sequences). The putative mutation ratio at G_0 was found to be 70.6%, as given by CrispRVariants analysis. Finally, we randomly selected and inbred a *bwb*-mutant strain among the multiple lines generated during this experiment, carrying mutant alleles with a -7 bp and -1 bp deletions at the sgR-Y-E2-01 targeted site (Figure S2). This newly created heteroallelic mutant strain was therefore named *ChYellow*$^{07/01}$, which has been maintained for more than 15 generations (phenotype shown in Figure 1D). These results demonstrate that mutations introduced by CRISPR technology are highly stable in *C*. *hominivorax*.

Mutational inheritance is dependent on Cas9 and sgRNA concentration

We then asked if the CRISPR/Cas9 genome editing efficiency is modulated by the final concentration of the delivered Cas9 RNP complexes into the screwworm embryos. To address this, *C*. *hominivorax* embryos were injected with a high (500 ng/µl) and a lower (360 ng/µl) concentration of Cas9 protein pre-assembled with sgRNAs against the *ChY* gene (Table 1). A single microinjector operator carried out all experiments using the same injection procedure in order to ensure the reproducibility of the technique. A lower proportion of injected embryos developed into adults with the high Cas9 concentration (9.5%; *n* = 38/400) compared with the lower concentration (16.9%; *n* = 63/373), or buffer alone (19.4%; *n* = 62/320), which suggested that the Cas9 protein might have some toxic effects at high concentrations. However, a relationship between the RNP concentration used and the mutagenesis rate was obtained as the proportion of flies with a mosaic *bwb* phenotype increased from 55.6% (*n* = 35/63) with the lower concentration to 73.7% (*n* = 28/38) with the higher Cas9 RNP concentration (Table 1). Furthermore, flies that developed from the embryos injected

with high Cas9 RNP concentration frequently showed larger body areas of low pigmentation (Figure S6). This observation indicated that biallelic mutations are favored when higher concentrations of RNP are used.

Table 1. Effect of CRISPR/Cas9 RNPs concentration on the screwworm survival, mutagenesis and germline transmission.

Cas9:sgRNA (ng/µl)	Injected embryo	Hatched larvae	Pupae	Adult Survivors at G_0			*bwb* mosaic at G_0			*bwb* transmission to	
				Female	Male	Total	Female	Male	Total	Founders	Inheritance
Control	427	323 (75.6%)	157 (36.8%)	76 (17.8%)	67 (15.7%)	143 (33.5%)	0	0	0	-	-
Mock	320	192 (60.0%)	71 (22.2%)	29 (09.1%)	33 (10.3%)	62 (19.4%)	0	0	0	-	-
360:145	373	180 (48.2%)	82 (21.9%)	29 (07.8%)	34 (09.1%)	63 (16.9%)	15 (23.8%)	20 (31.7%)	35 (55.6%)	8/10 (80.0%)	38.4 ± 4.3%
500:200	400	137 (34.2%)	48 (12.0%)	23 (05.7%)	15 (03.7%)	38 (09.5%)	18 (47.4%)	10 (26.3%)	28 (73.7%)	06/8 (75.0%)	81.9 ± 14%

Hatched larvae ratio is given as percentage relative to the number of embryos; ***Pupae*** ratio is given as percentage relative to the number of adult survivors; ***Founders*** refers to the number of G_0 *bwb* mosaic males capable of transmitting Cas9-derived mutant alleles to G_1 generation after backcrossed with the females of the former established *ChYellow* $^{07/01}$ strain, and; ***Inheritance*** is given as average ± SEM of G_1 biallelic *bwb* mutants obtained from the backcrossings between G_0 mosaic males and *bwb* females from the *ChYellow* $^{07/01}$ mutant strain (individual result from each cross are shown in Table S3).

Since embryos were injected at the posterior end prior to pole cell formation, it was anticipated that this would lead to mutations of the *ChY* locus in the germline. In order to estimate the level of induced germline mutagenesis, mosaic G_0 males were crossed with virgin *bwb* females, from the earlier established *ChYellow*$^{07/01}$ strain, and their offspring screened for the *brown body* phenotype. While six out of eight (75%) viable ovipositions derived from flies injected with the high Cas9 concentration revealed *bwb* mutants, 80% of the crossings resulted in bwb progeny for the lower concentration. From the crosses with the G_0 males using the lower Cas9 concentration injections, 18.5 to 49.3% (Avg. ± SEM; 38.4 ± 4.3%) of the offspring had a *brown body* phenotype. Flies derived from the high concentration experiment produced *bwb* mutant progeny at a frequency ranging from 13 to 100% (81.9 ± 14%; Table 1 and Table S3). Hence, it appears that injecting embryos with a high Cas9 concentration led to a higher frequency of germline mutations at the targeted locus.

Disruption of transformer gene causes masculinization of XX Screwworm flies

Transformer (*tra*) is the master gene that controls sex determination in many Diptera species, including blowflies (Scott et al. 2014b). Briefly, *tra* transcripts in *C. hominivorax* are sex-specifically spliced such that only the female variant (*tra*^{F})

encodes a full-length functional TRA protein, which presumable triggers the female development pathway by default. On the other hand, an unknown Y-linked male-determining gene (*M*) prevents the splicing of the male-specific exon M1, which contains several in-frame stop codons that impairs the translation of a functional TRA protein, and thus the female pathway of development is prevented and male development occurs (Li et al. 2013). Previous RNA interference (RNAi) experiments in *L*. *cuprina*, *Lucilia sericata* and *Cochliomyia macellaria*, showed that females can be "*reprogramed*" to develop as XX males by knocking down the expression of the *tra* mRNA (Concha and Scott 2009; Li et al. 2013).

In this context, we targeted *Chtra* to determine if the CRISPR protocol established for *ChY* could be used for efficient Cas9-mediated gene disruption of another locus in the screwworm genome. We carried out microinjections in *C*. *hominivorax* embryos targeting the exon-1-intron-1 boundary region of *Chtra* gene (Figure 2A). In total, 24 viable adults developed from 260 injected embryos (9.2% survival, similar to our previous results with *ChY*), of which 14 were phenotypically normal males. Out of the 10 surviving G_0 females, four showed normal female characteristics while six (60%) developed intersexual phenotypes that were characterized by the presence of an abnormal terminalia with male-like structures, but a female interocular width (Figure 2B). The intersex phenotype appeared as partially to fully transformed ovipositors into male genitalia and, upon dissection, these flies also exhibited abnormal reproductive tissues, including the absence of female structures and/or atypical ovary development (Figure 2B, last row of the panel). To confirm that the male-like structures found in these G_0 females were due to somatic mutations in the *Chtra* gene, 3 mosaic flies showing different degrees of transformation were selected (intersex individuals showed in Figure 2B), DNA extracted and analyzed by T7EN1 assay (Figure S4). Results showed band migration patterns consistent with the expected Cas9 cleavage from the experimental design, while no activity in the control wildtype fly was observed, revealing that these individuals carried indels at the targeted region of *Chtra*. The spectrum of mutated alleles induced by Cas9 was then accessed via Sanger sequencing of the same PCR amplifications used in the T7EN1 assay (Figure 2C and S7). Sequenced clones (n = 22) confirmed the presence of indels in *Chtra* gene, which were mostly small deletions (Δ -6 to -13 bp), although some

medium deletions (Δ -37 to -54 bp) were also found. The mutagenesis efficiency at G0 was predicted to be 72.7%, as given by CrispRVariants analysis.

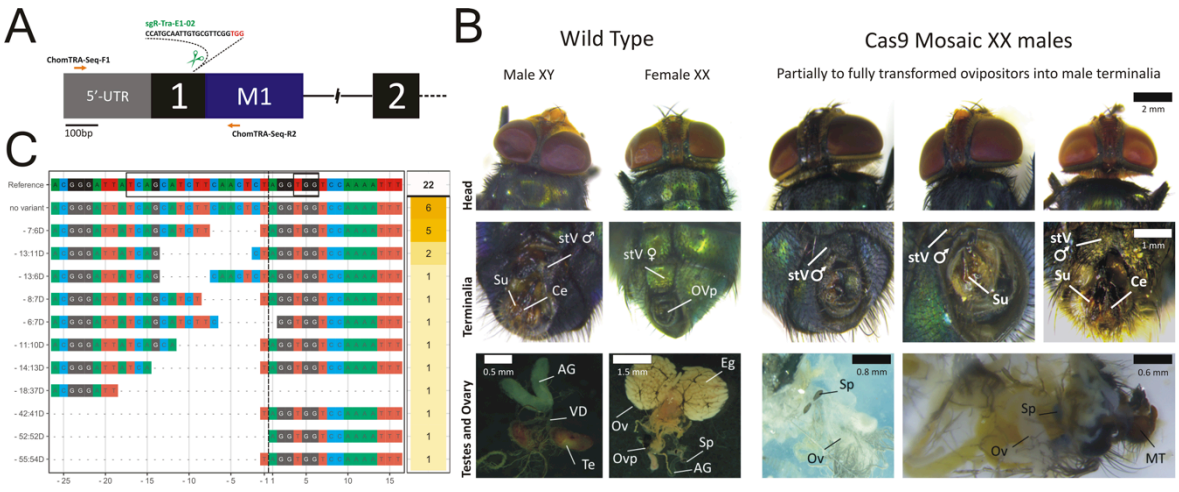

Figure 2. Knockout of screwworm *tra* loci (*Chtra*) causes masculinization of XX flies. **(A)** Schematic of the 5'-proximal *Chtra* gene organization and CRISPR strategy used for knockouts. **(B)** Intersexed phenotypes of *Chtra* G_0 mosaic mutants (leftmost) in comparison with wildtype male and female (rightmost). Adult flies head (above), genitalia (middle) and dissected reproductive tissue (below) are shown. Cas9-disruption of *Chtra* gene leads to the development of abnormal genitalia and ovaries in G_0 females, ranging from partially to fully transformed ovipositors into male terminalia. **(C)** CrispRVariants (Lindsay et al. 2016) plots of *Chtra* Cas9-induced mutant alleles found in G_0 flies in comparison with the wildtype sequence. Vertical black dashed line indicates the predicted position of Cas9 cut site (see Figure S7 for a more comprehensive alignment). Number of sequenced clones per allele is shown in the right yellow box. Abbreviations used: stV, sternite V; Su, sustylus, Ce, cerci; AG, accessory gland; VD, vans deferens; Te, testis; MT, male terminalia; OVp, ovipositor; OV, ovary; Eg, eggs, and; Sp, spermathecal (as described by Spradbery and Sands 1976).

DISCUSSION

Developing efficient gene manipulation strategies in non-model insects is still a challenge that slows the investigations on their unique biology and natural adaptations. Additionally, the implementation of new genetic strategies to manage economically and medically important insect pest species also requires the development of efficient genome manipulation tools and protocols. In the present study, we described CRISPR/Cas9 site-directed and heritable mutagenesis protocols for *in vivo* functional analysis of candidate loci in the screwworm fly, *C. hominivorax*. To our knowledge, this is the first demonstration of a directed mutagenesis induced by CRISPR/Cas9 in *Cochliomyia*. The methods serve as

the basis for further functional genomic studies in *C. hominivorax*, as well in other calliphorids and related biological systems.

Until now, reverse genetic studies in calliphorids have been performed by RNAi-mediated loss-of-function experiments (Mellenthin et al. 2006; Concha and Scott 2009; Li et al. 2013), while homozygous transgenic strains have been developed by piggyBac-mediated germline transformation (Li et al. 2014; Yan and Scott 2015; Concha et al. 2016). Although these resources provided means to functionally investigate genes of interest in these species, they have limitations that CRISPR/Cas9 genome editing can help to address. For example, directed injection of double-stranded RNA (dsRNA) into embryos is prone to affect several off-target transcripts (Smith et al. 2017) and its effects are transient unless stabilized as part of a transgenic hairpin construct (Brown et al. 2003). On the other hand, CRISPR-mediated genome editing usually generates stronger and more consistent phenotypes and it is prone to reduce off-target effects (Evers et al. 2016; Housden and Perrimon, 2016). In contrast to RNAi, genomic modifications by piggyBac are heritable, but often display a lower frequency of germline transformation in comparison with CRISPR induced knockouts. Another concern of transposon-mediated transformations is that the transgene is randomly integrated into the genome, which in turn requires individual strains to be evaluated with respect to their fitness. As genome editing by CRISPR is site-directed, it offers the opportunity to decide where the edits will be performed in the genome. Furthermore, in the case of transgenic sexing strains designed for use in potential SIT programs, it is often desirable to stabilize the genetic construct in the genome of the insects by removing one of the piggyBac arms, such that it is impossible for the construct to change its position in the genome. The potential use of CRISPR to create transgenic lines by performing directed knock-ins would be helpful to avoid this technical issue. Here, we reported a high frequency of CRISPR/Cas9-meditated mutagenesis of the *ChY* and *Chtra* genes, particularly at the high concentration of Cas9/sgRNA complex. Furthermore, a high proportion of surviving G_0 showed a mosaic phenotype, suggesting that mutations had been introduced at both copies of the targeted genes. CRISPR/Cas9 G_0 mosaic mutants provide the benefit of allowing the functional study of genes that are essential for development or normal cell function,

for which a homozygous knockout line would be lethal, as there would be "*escapers*" since not every cell in the organism is edited.

We found that embryonic injection with a high concentration of Cas9 protein (500 ng/µl) preloaded with sgRNAs promotes a high mutagenesis frequency at the *yellow* locus in *C. hominivorax*. However, the survival frequency of injected embryos was slightly lower with the high concentration of Cas9, as also observed for other insect species (Kistler et al. 2015; Li et al. 2017b, 2017a). Hence, we believe that for most targets a mid-range concentration (400 ng/µl) would be suitable to balance survival rate and mutagenesis efficiency, despite the need of increasing the number of injected embryos and replicates for statistical comparisons. A high Cas9 concentration might be desirable when targeting recessive autosomal markers, as it favors biallelic hits and thus a mutant phenotype in the surviving G_0. The need for Cas9 protein could be avoided by making transgenic strains that express Cas9 either in the germline for generating stable mutant lines or in all cells for screening G_0 for a mutant phenotype. For example, *Drosophila* strains that produce Cas9 in the germline have been used for efficient gene editing (Kondo and Ueda 2013; Ren et al. 2013; Port et al. 2015). In addition to generating random indels at the targeted gene as shown in this study, specific nucleotide changes can be made if repair is mediated by the homology-directed repair (HDR) mechanism with a supplied repair template. For example, Aumann et al. (2018) recently used short single-stranded DNA donor (ssODN) to introduce point mutations in the genome of the Mediterranean fruit fly, *Ceratitis capitata*. The authors described a highly efficient protocol for CRISPR knockin in this invasive insect pest, where ~86% of G_0 fertile females produced mutant offspring and transmitted the edited allele to 71 to 79% of their progeny. Therefore, a logical next step would be to establish this approach in *C. hominivorax* to promote specific changes to genes of interest. This could be done, for instance, to delete TRA/TRA2 biding sites that are hypothesized to be important for sex-specific splicing of *tra* transcripts in blowflies (Concha et al. 2009; Li et al. 2013).

Currently, the only markers available for germline transformation of *C. hominvorax* are fluorescent protein genes driven by a strong constitutive promoter (Concha et al. 2012, Concha et al. 2016). While they greatly facilitate identification of transgenic flies, the markers may not be appropriate if an experiment requires low-

level background fluorescence. For such experiments the *bwb* mutant strains developed in this study could be useful recipients for germline transformation with vectors containing *ChY*$^{+}$ as the marker gene. Transgenic flies would be easily identified through rescue of the wildtype body color.

We further demonstrated that the CRISPR protocol developed for the *yellow* locus is suitable for generating knockouts in other regions of the screwworm genome, by targeting *transformer* (*tra*; Figure 2A). This is a key gene required for normal female development in many dipterans (Schutt and Nothiger 2000; Verhulst et al. 2010). Therefore, we hypothesized that Cas9-induced frameshifts in the *C. hominivorax tra* gene would lead to masculinization of G_0 females, which is similar to what was observed with RNAi knockdown experiments in the closely related species *C. macellaria* (Li et al. 2013). We found that Cas9-mediated mutagenesis of the exon1-intron1 boundary of *Chtra* led to the development of intersexual flies showing a normal female interocular width, but with anomalies in their genitalia and reproductive tissues that were male-like (Figure 2B). Overall, out of 10 surviving females, obtained after Cas9-sgRNA injections, six presented different degrees of masculinization, including a single female with a fully developed male terminalia (Figure 2B, last column of the panel). All adult males developed from injected embryos showed normal phenotypes, indicating that mutations in *Chtra* are not hazardous for male development. Yet, it is important to notice that any fully transformed XX males would be undetectable in our results due to the lack of sex-linked markers for *C. hominivorax*, which makes impossible the screening for true XX and XY individuals. As expected by its high conservation between closely related species, the results confirmed that *tra* is required for normal female development in *C. hominivorax*, and suggested that the *Chtra* gene could be a potential target for future Cas9-mediated gene drive strategies targeting female development. CRISPR-based gene drive strains could be evaluated in the future for population suppression of these important pests of livestock (Hammond et al. 2016; Scott et al. 2018; Kyrou et al. 2018). Concha and Scott (2009) found that some fully transformed XX *L. cuprina* flies are fertile, as it was also described by Zhao et al. 2018 for the Oriental fruit fly, *Bactrocera dorsalis*. These observations implicate that the disruption of *tra* is sufficient to exchange not only sex-specific morphology, but also behavior in these species. In this context, both XY and

fully transformed XX flies would contribute to population suppression. Although the benefits of such strategy remains to be evaluated, for the present, a CRISPR/Cas9 system targeting the *tra* locus in *C*. *hominivorax* would be desired to develop steady male-only strains for a more efficient SIT program (Kandul et al. 2019). For instance, Rendón et al. (2004) observed that the field release of unisexual sterilized medflies was several-fold more advantageous in relation to bisexual releases. Therefore, for a potential sterile male field release, males and intersexes could be generated by crossing strains that express Cas9 in somatic cells and sgRNAs constitutively. The intersexes would be expected to be sterile. A disadvantage of this approach is that a large number of virgin females would need to be collected from one strain to set the cross (in order to maintain the line at the laboratory), which could be impractical in a production facility. An alternative would be to regulate Cas9 expression using the tetracycline transactivator (tTA), which is easily repressed by adding tetracycline to the diet. This would be similar to the previously generated tetracycline repressible female lethal strains (Li et al, 2014, Concha et al, 2016, Yan et al 2015). In addition to *tra*, it would be desirable to evaluate other sgRNAs against the *doublesex* (*dsx*) and *fruitless* (*fru*) genes, which are also essential for female development (Scott et al. 2014b). The highly conserved female-specific exon of *dsx* in particular appears to be a promising target for gene drive (Hammond et al 2018).

Perhaps one of the most interesting and complex questions in Calliphoridae evolution is how ectoparasitism (obligatory and facultative) arose independently in some lineages of the family, including the ones that led to the *C*. *hominivorax* species (Stevens et al. 2006; Hall et al. 2016; Junqueira et al. 2016). The major transition from a free living to a parasitic organism is expected to require a number of morphological, reproductive, and physiological adaptions. In the case of calliphorids, we are particularly interested in molecular adaptations towards host seeking by chemical signal transduction as chemoreception plays an essential role during specialization, behavior, and niche establishment (Hansson and Stensmyr 2011). In contrast to their closely related cousins that seek dead animals, the parasitic calliphorids must seek out live warm-blooded animals. The availability of assembled genome sequences together with the establishment of the CRISPR technology, described here, provide the foundation for functional investigations on genomic regions linked to the evolution of

natural traits in these parasitic flies. This opportunity provides the nexus to interrogate evolutionary aspects of *C. hominivorax*, which could translate in the development of new environmentally friendly management strategies for parasitic calliphorids, and possibly for other arthropod parasites and disease vectors.

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SUPPLEMENTAL MATERIAL

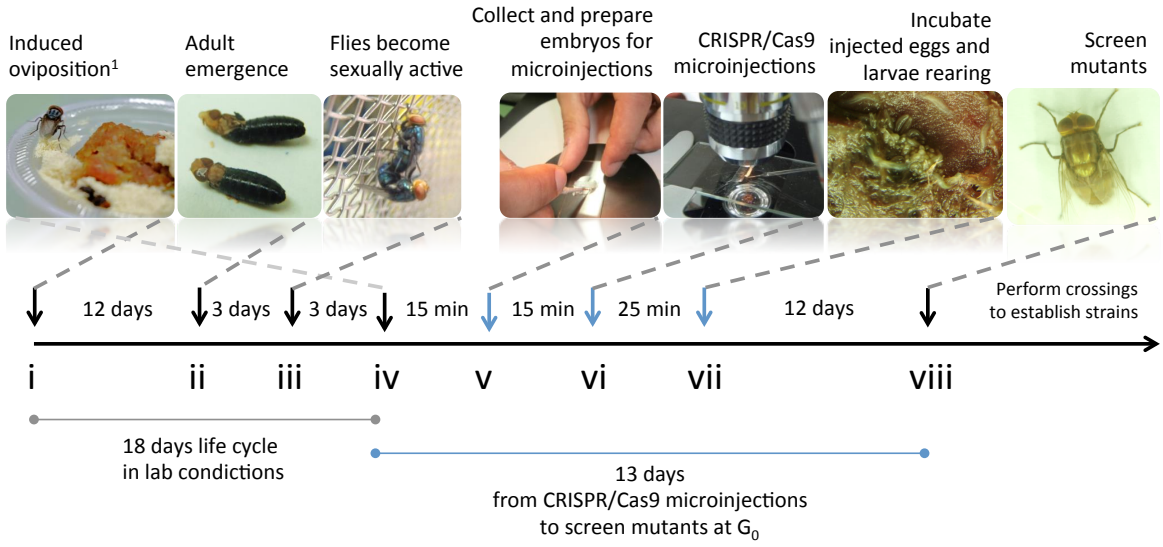

Figure S1. Schematic timeline of *Cochliomyia hominivorax* rearing and CRISPR/Cas9 microinjection experiments. The New World Screwworm fly takes 18 days to mature from eggs to adult in laboratory conditions (gray line: i to iv). Females are ready to lay eggs six days after emergence (i and iv). First instar larvae hatch ~12 hrs later, passing through three larval instars before dropping to the ground to pupate. Adults emerge 12 days after oviposition (ii) and females become sexually receptive three days after emergence (iii). For microinjections, fresh screwworm embryos are collected (iv), dissociated, aligned and dehydrated (v). Microinjections of CRISPR/Cas9 cocktail are performed into the posterior end of the pre-blastoderm embryos within the first 40 min of development (vi). After hatching, immatures are reared to develop until adulthood (vii) and emerged injected G_0 adults are subsequently screened for Cas9-induced mutations (viii). This entire experiment takes approximately 13 days to complete (blue line: iv to viii). [1] Photograph by Jorge Herrera.

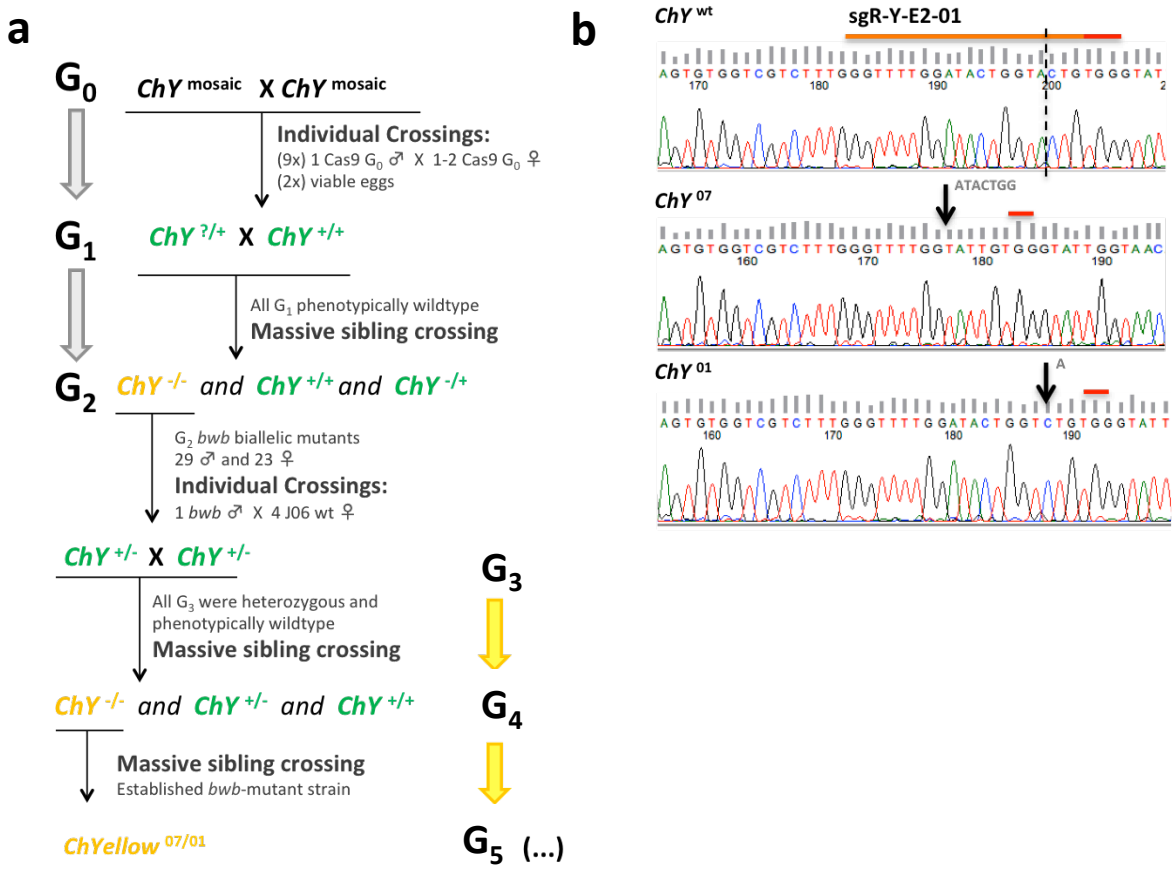

Figure S2. Crossing scheme used to establish the biallelic *bwb*-mutant strain of the screwworm fly. (a) Mosaic *bwb*-mutant flies (Fig. 1B, second column) were obtained after microinjections of Cas9 RNPs against the *ChYellow* loci. Nine individual crossings were made aiming to evaluate the inheritance capability of the Cas9-induced mutations in the *ChY* gene, but only 2 resulted in viable ovipositions. All G_1 flies were phenotypically wildtype, presumably heterozygous for the *bwb* mutation. As the *bwb* mutation is recessive and autosomal in *C. hominivorax*, only mutations in both copies of the *ChY* gene will result in the unpigmented body phenotype. Thus, massive sibling crossings were carried out, and biallelic *bwb* mutant flies were obtained at G_2 generation (Fig. 1B, last two columns). A *bwb* male was randomly selected and backcrossed to wildtype females (yellow arrows). The offspring were let to interbreed freely in cages and the obtained biallelic *bwb*-mutants used to establish the strain *ChYellow*$^{07/01}$, currently being maintained at the COPEG biosecurity plant in Panama-PA. **(b)** Molecular genotyping of *ChYellow*$^{07/01}$.

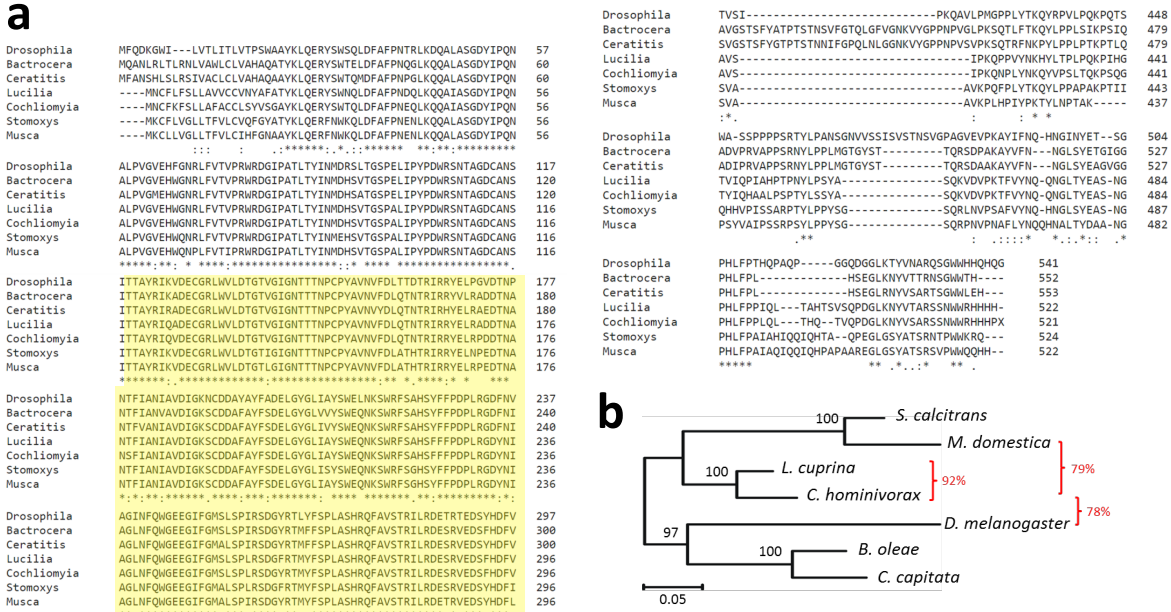

Figure S3. Orthology analysis of the *yellow* gene found in Schizophora species. (a) Multiple sequence alignment of yellow protein from *Drosophila melanogaster* (NP_476792.1), *Bactrocera oleae* (XP_014092322.1), *Ceratitis capitata* (XP_004521097.1), *Lucilia cuprina* (XP_023305007.1), *Cochliomyia hominivorax* (M.J.Scott, unpublished), *Stomoxys calcitrans* (XP_013102593.1) and *Musca domestica* (XP_011290952.1). Major Royal Jelly Protein (MRJP; pfam03022) conserved domain is highlighted in the yellow box **(b)** Evolutionary relantionship of yellow protein reconstructed by the Maximum Likelihood method under the JTT+G+I substitution model. Node supports were estimated under 100 replicates of bootstrap. Sequence identity between key species are shown in red.

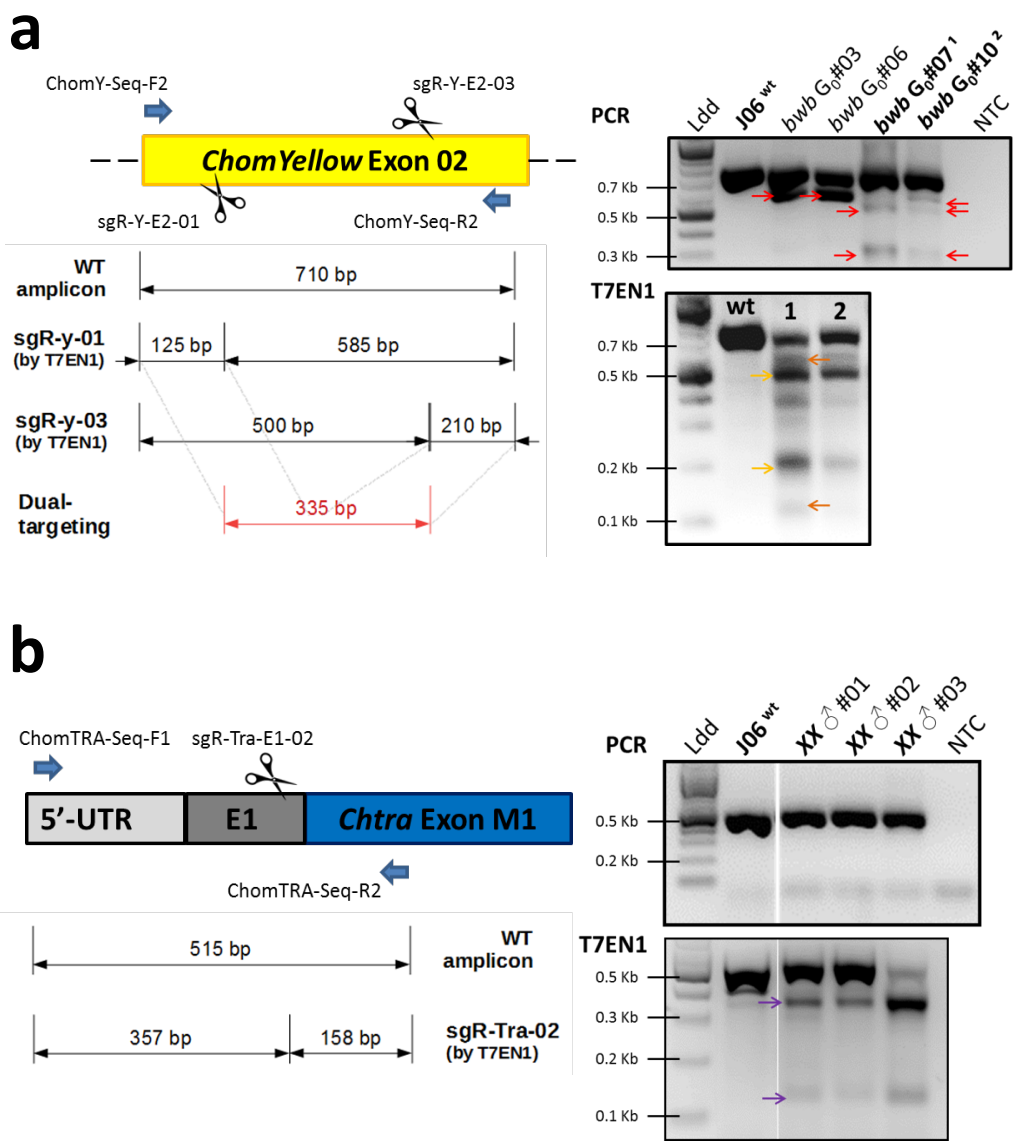

Figure S4. T7 endonuclease 1 assay (T7EN1) of Cas9 targeted sites in the screwworm genome. (a) Schematic of CRISPR strategy used to knockout the *ChY* loci (leftmost), showing the positions of each sgRNAs (scissors), PCR genotyping primers (blue arrows), the PCR amplicon size for wildtype (wt) and the expected band migration after the T7EN1 assay. (rightmost) PCR amplifications of *ChY* targeted site on J06 wt and selected *bwb* mosaic flies obtained at G_0 (above). Putative medium to large deletions (red arrows) were detected in these amplifications, presumably due to the *dual-targeting* approach (see alleles in Figure 1C and S5). Only crosses made with the male flies #07 and #10 resulted in viable G_1 eggs (see Results), thus these two flies were submitted to the T7EN1 assay (bellow), which revealed the presence of indels at the specific Cas9 targeted sites of the used sgRNAs: sgR-Y-E2-01 (orange arrows) and sgR-Y-E2-03 (yellow arrows). **(b)** (leftmost) Schematic of CRISPR strategy used to knockout the *Chtra* locus and expected band migration for wt and after T7EN1. (rightmost) PCR amplifications of *Chtra* targeted site (above) and results obtained by T7EN1 assay (bellow) for the intersexed flies showed in Figure 3B. Band migration patterns consistent with the expected Cas9 cleavage using the sgRNA sgR-Tra-E1-02. Other abbreviations used: Ldd = Ladder; NTC = non-template control; Kb = Kilo bases.

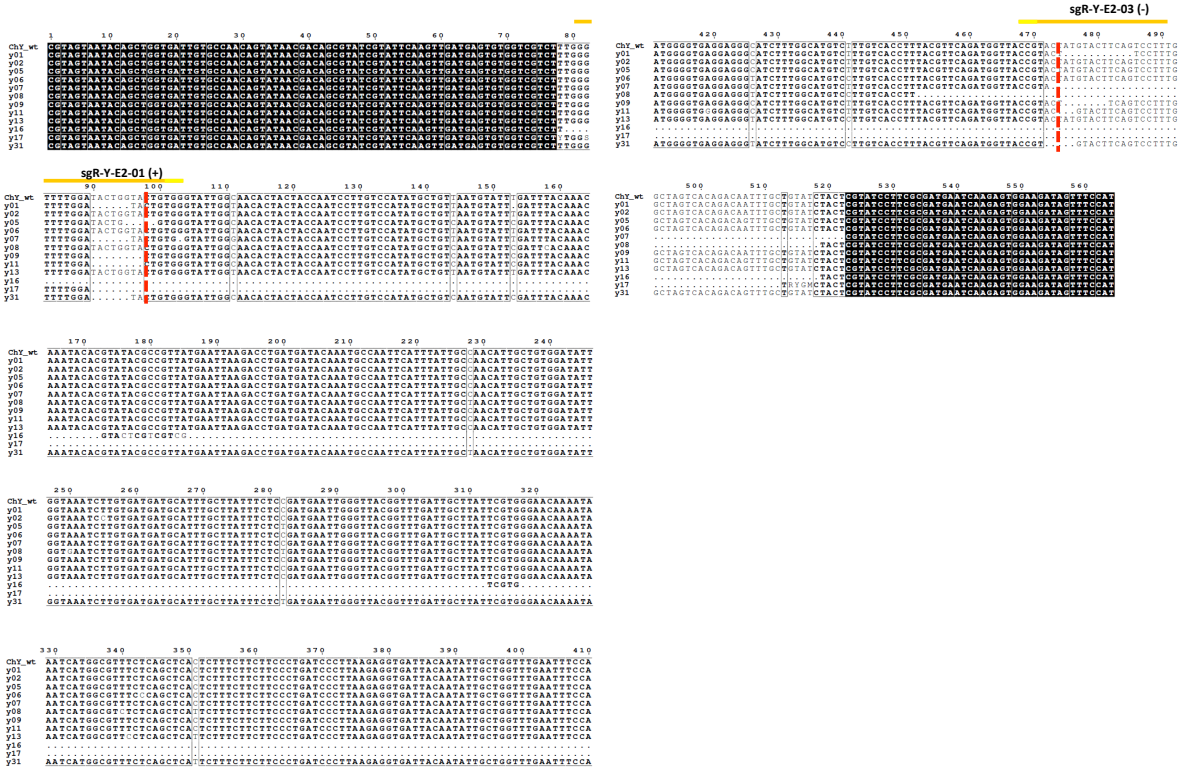

Figure S5. Multiple Sequence Alignment of *bwb*-mutated alleles in *C. hominivorax* introduced by Cas9. Sequence variants recovered from G_0 flies were mapped against the wildtype reference sequence (ChY_wt, in the alignment) using MUSCLE algorithm (Edgar, 2004) implemented on MEGA7 (Kumar et al. 2016), with default options. Alleles were collapsed in "*haplotypes*" (y#, in the alignment) using the online tool ALTER (Glez-Peña et al. 2010) and visualized using ESPript v.3.0 (Robert and Gouet, 2014; nucleotides in black boxes represent *%Equivalent* ≥ 0.7). Positions of the sgRNAs used in the microinjections experiments are highlighted in orange and their respective PAM motifs in yellow. The Cas9 predicted cut sites (3-nt upstream of the PAM) are shown in red-dashed lines. We were able to recover 2 "*haplotypes*" (y16 and y17), represented by 9 clones (Fig. 1C), showing a large deletion (~360bp) between both sgRNAs target sites, indicating that *CRISPR dual-targeting approach* can be used for molecular genotyping of screwworm Cas9-derived mutants, although optimization is still required.

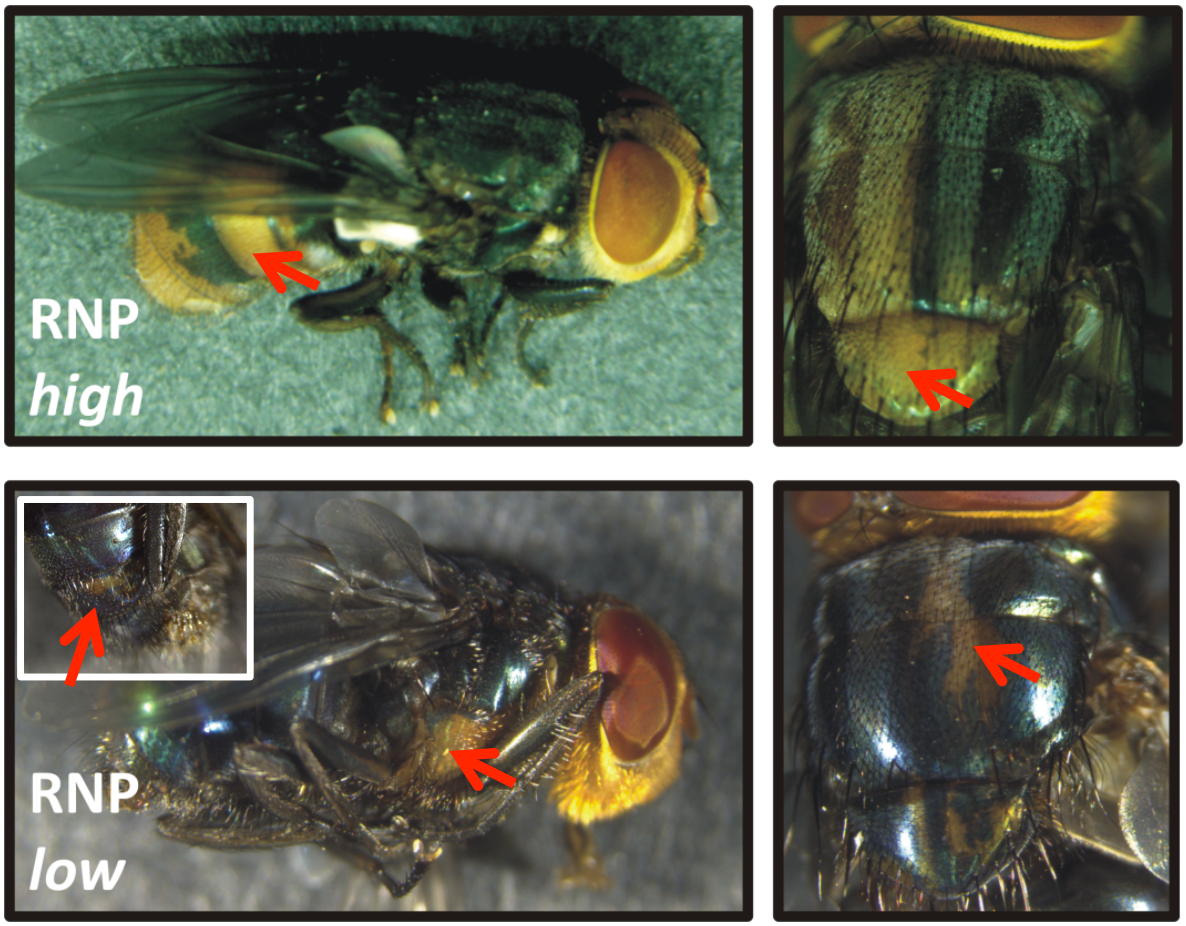

Figure S6. Phenotypic variation between mosaic *bwb* screwworm flies. Adult mutant phenotypes of individuals injected with the high (500 ng/ul) concentration of the RNPs frequently showed a larger unpigmented body area than those injected with the low (360 ng/ul) concentration (indicated by red arrows).

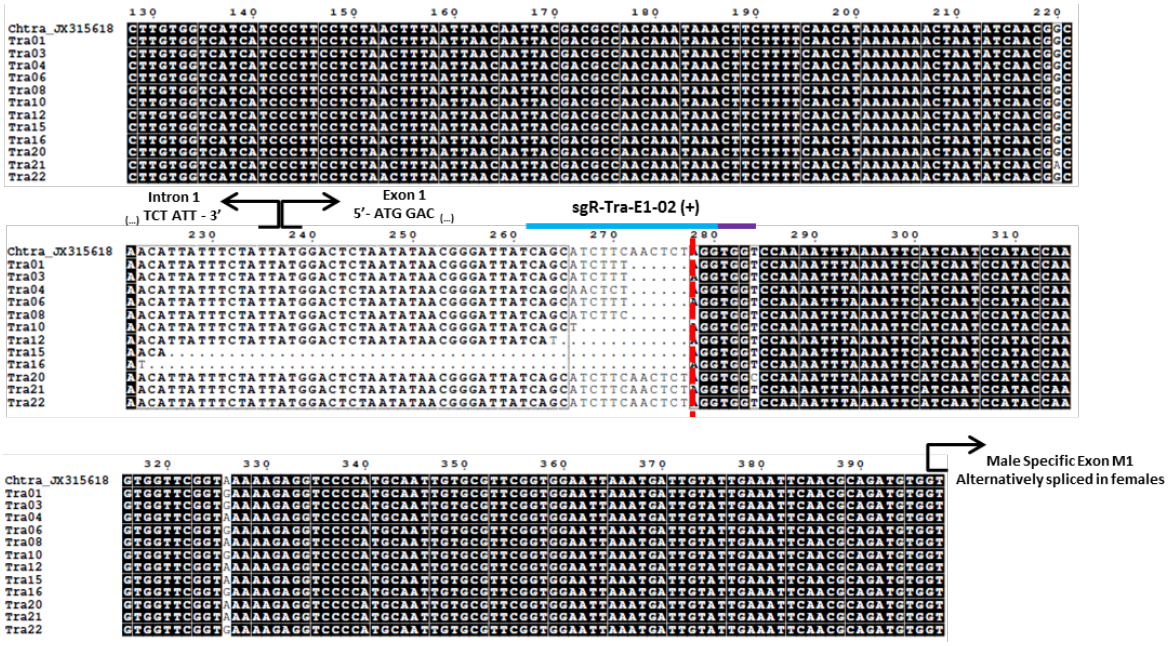

Figure S7. Multiple Sequence Alignment of *tra*-mutated alleles in *C. hominivorax* genome introduced by Cas9. Sequence variants recovered from intersexed G_0 flies (Fig. 3B) were mapped against the wildtype reference sequence (Chtra_JX315618, in the alignment) using MUSCLE algorithm (Edgar, 2004) implemented on MEGA7 (Kumar et al. 2016), with default options. Alleles were collapsed in "*haplotypes*" (TRA#, in the alignment) using the online tool ALTER (Glez-Peña et al. 2010) and visualized using ESPript v.3.0 (Robert and Gouet, 2014; nucleotides in black boxes represent *%Equivalent* ≥ 0.7). Position of the sgRNA used in the microinjections experiments is highlighted in blue and the PAM motif in purple. The Cas9 predicted cut sites (3-nt upstream of the PAM) are shown in red-dashed lines. Black arrows indicate the genomic position of *Chtra* Intron 1, Exon 1 and the start of the male specific exon M1.

Table S1. Sequences of the single guide RNAs (sgRNAs) used in this study. The sgRNAs were designed by using the standalone version of CRISPOR tool (Concordet and Haeussler 2018). Potential off-targets (see alignments bellow) were evaluated in the context of *C. hominivorax* draft assembly (A.C.M. Junqueira, unpublished) and the complete genome sequence of *L. cuprina* (Anstead et al. 2015). We only considered sgRNAs that have potential off-targets with more than 3 mismatches in total, with the maximum amount of mismatches present on the 5 – 10 first bases of the sgRNA directly upstream to PAM motif, which constitute the so called "*seed*" region (Andersson et al. 2015; Zheng et al. 2016). Syntheses of sgRNAs were performed via PCR as described by Bassett and Liu (2014), using the primers sgR-Specific-T7-FWD (5'- GAA ATT AAT ACG ACT CAC TAT A(**GG**) [***specific sgRNA sequence without PAM***] TTG GGT TTT AGA GCT AGA AAT AGC -3') and sgR-Universal-REV (5'- AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATT TCT AGC TCT AAA AC -3').

Species	Target site	Guide Name	Direction	Protospacer + PAM motif	Length	Potential off-targets for 0-1-2-3-4 mismatches	mitSpecScore
Cochliomyia hominivorax	*Yellow* (*ChY*)	sgR-Y-E2-01	+	(GG)TTTTGGATACTGGTACTG TGG	20 nt	0-0-0-0-4 [a]	89
		sgR-Y-E2-03	-	AAGGACTGAAGTACATAGTA CGG	20 nt	0-0-0-0-5 [b]	69
	transformer (*Chtra*)	sgR-Tra-E1-02	+	CCATGCAATTGTGCGTTCGG TGG	20 nt	0-0-0-0-3 [c]	92
Lucilia cuprina	*Yellow* (*LcY*)	Lc-y-sgRNA2	+	AGCATAGGGGCAAGGAT TGG	17 nt	0-0-0-1-1 [d]	100
		Lc-y-sgRNA1	+	TGTTTGTAACGGTTCCCAGG TGG	20 nt	0-0-0-0-1 [e]	100

Table S2. Specifications of the genotyping primers used in this study. Primers used to sample the allele variants introduced by the Cas9 RNPs on the screwworm and blowfly targeted loci. PCRs were followed by molecular cloning and Sanger sequencing. Sequenced reads were analyzed by CrispRVariants package v.1.8.0 (Lindsay et al. 2016).

Species	Targeted site	Primer Name	Sequence (5' → 3')	Length	Ta	Amplicon Size
Cochliomyia hominivorax	***Yellow* (*ChY*)** 5'-end Exon 2 (Figure 1A)	ChomY-Seq-F2	GCC CTA ATA CCG TAC CCC GA	20 nt	59.4°C	710 bp
		ChomY-Seq-R2	AGA AGA ATG CCA GCA ACC GA	20 nt		
	***transformer* (*Chtra*)** 5'-UTR to Intron M1 (Figure 3A)	ChomTRA-Seq-F1	GTC AGC AGC AAA GAT CTG TCA	21 nt	51.2°C	515 bp
		ChomTRA-Seq-R2	AAT CCC AAC AGT ATG CTT	18 nt		
Lucilia cuprina	***Yellow* (*LcY*)** 5'-end Exon 2	Lc-y-g1F	CTT ACA TCA ATA TGG ATC ACA GTG TAA CC	29 nt	55.6°C	283 bp
		Lc-y-g1R	AAT GTA TTG GCA TTT GTA TCA TCA GCT C	28 nt		
	3'-end Exon 1 (Figure 2A)	Lc-y-g2F	GAA CGT TAT AGT TGG AAT CAG TTG GAT T	28 nt	52.2°C	431 bp
		Lc-y-g2R	GAA TCC TCA TCA TCA ATA ATT GTA TCA GAC A	31 nt		

Table S3. Effect of Cas9 RNPs concentration on germline transmission of *bwb* mutation. For each microinjection experiment using a low (360ng/ul) or high (500ng/ul) concentration of Cas9 protein, 10 mosaic males were randomly selected and tested for their founder habilities by individually backcrossing them with virgin *bwb* females. The G1 offspring were screened for the presence of biallelic *bwb* mutants, revealing germline transmission of the mutated allele.

Backcrossing to *bwb* strain	G0 ♂ founder generated with:	
	Cas9 360ng/ul	Cas9 500ng/ul
Cage 01	56 / 139 (40.3%)	Infertile eggs
Cage 02	0 / 120 (00.0%)	11 / 83 (13.3%)
Cage 03	0 / 131 (00.0%)	78 / 96 (81.3%)
Cage 04	47 / 125 (37.6%)	Infertile eggs
Cage 05	78 / 173 (45.1%)	126 / 126 (100%)
Cage 06	71 / 144 (49.3%)	117 / 121 (96.7%)
Cage 07	32 /173 (18.5%)	120 / 120 (100%)
Cage 08	77 / 163 (47.2%)	0 / 112 (00.0%)
Cage 09	85 / 178 (47.8%)	0 / 145 (00.0%)
Cage 10	31 / 147 (21.1%)	118 / 118 (100%)
Avg ± SEM	**38.4 ± 4.3%**	**81.9 ± 14%**

CHAPTER 2

Molecular and Functional Characterization of the Olfactory Co-receptor Orco of *Cochliomyia hominivorax* [4]

Abstract The evolution of obligatory ectoparasitism in Calliphoridae family (blowflies) has challenging the scientific community for over a century, but surprisingly the genetics underlying such life strategy remains largely unknown. Like in other insects, blowflies rely on volatiles cues to find hosts for oviposition. Therefore, we hypothesize that olfaction may have played a critical role in the adaptive transition from a free-living to a parasitic species within the group. Hence, we performed the characterization, evolutionary and expression profiling of the Olfactory Receptor Co-receptor (Orco) in the New World Screwworm fly, *Cochiomyia hominivorax*, one of the only obligatory ectoparasitic Screwworms in the world. Orco is an ubiquitous co-receptor for all Odorant Receptors (ORs), a variable receptor family essential for many olfactory-evoked behaviors in insects. Screwworm Orco (*ChomOrco*) is highly conserved within Diptera, and it's evolving under a strong purifying selection regime. Transcription of *ChomOrco* is particularly high in adults, broadly expressed in olfactory and other chemosensory appendages, and it's linked to morphological, developmental and behavioral aspects of *C. hominivorax*. We used CRISPR/Cas9 targeted mutagenesis to interrogate *ChomOrco* for its consequences on Screwworm behavior. Two-choice bioassays revealed that Orco mutants display a disrupted flight orientation and decision-making, no longer responding to floral-like and hosts' volatiles. The results imply that ORs are not only essential for foraging, but host-seeking behaviors in *C. hominivorax*. The data presented here, corroborates with the hypothesis that OR-family evolution is linked to host preference in Screwworm, providing us the basis for novel functional, ecological and evolutionary studies in blowflies.

Keywords: Functional Genomics, Olfactory Receptor Co-receptor, Orco, Or83b, Olfaction, Chemosensory Genes, RACE-PCR, Non-lethal genotyping, Genome Editing, CRISPR/Cas9, Myiasis, Ectoparasitism, Obligatory Parasitism, Calliphoridae, Blowflies, Screwworm flies.

[4] This chapter represents a draft manuscript in preparation by the authors. (See Appendix D for more details).

INTRODUCTION

Identifying genomic regions that underlie the adaptive radiation of insects is a major goal in evolutionary biology. In particular, the emergence of a pest insect offers us an unparalleled opportunity to investigate the genetic basis of adaptive traits and how species evolve to occupy novel ecological niches. As once said by Grimaldi and Engel (2005), "*Calyptratae flies have redefined the "art" of vertebrate parasitism, particularly the Oestroidea*(…)" [a superfamily that includes blowflies, botflies, fleshflies and relatives]. Within Oestoidea, the family Calliphoridae (blowflies) has received particular attention due to its diversity, which comprises over 1,500 described species worldwide (Pape et al. 2011). Commonly known as blowflies, the members of this family are frequently found foraging on plant inflorescences (Heath 1982; Brodie et al. 2016), as well as feeding and breeding on decaying organic matter and carcasses (Guimarães and Papavero, 1999; Amendt et al. 2004). Therefore, blowflies have not only an important role in nature as pollinators but also as recyclers of organic waste, in addition to their scientific importance in forensic (Krinsky 2019), veterinary (Hall and Wall 1995) and many aspects of medical sciences (Sherman et al. 2000; Hall et al. 2016; Junqueira et al. 2017). Although better known for these necro-saprophagous flies, the group spans an even great diversity of life-history strategies, including many forms of parasitism (examples are given by Kutty et al. 2010; McDonagh and Stevens, 2011; Marinho et al. 2012). In special, the emergence of the obligatory ectoparasitism is one of the most remarkable evolutionary events within the Calliphoridae family, which has been the subject for scientific debate for over a century (see the essay by Erzinclioglu, 1989). Molecular timescale phylogenetic studies have presented evidence that the obligatory ectoparasitic habit arose recently and independently multiple times after the explosive radiation of the family ca. 22 million years (Ma), deriving from a free-living necro-saprophagous ancestry (Stevens 2003; Wallman et al. 2005; Stevens and Wallman 2006; McDonagh and Stevens 2011; Junqueira et al. 2016). However, the genetic basis of such a life strategy remains largely unknown.

In an attempt to overcome this knowledge constraint, we propose to start studying the evolution of olfactory perception in blowflies by adopting the New World

Screwworm fly, *Cochliomyia hominivorax* (Coquerel, 1858), as a model species. Like in many other insects, Screwworm flies rely on olfactory clues to find suitable sites for oviposition, which in this case are volatile chemical compounds (odors) produced by wounded and vunerable warm-blooded vertebrates (Tomberlin et al. 2017). Gravid Screwworm females lay their eggs on dry margins of wounds and orifices, and after hatching, the larvae (maggots) infest and consume the host's living tissues for several days to complete its development (Alexander 2006; Hall et al. 2016). The resulting traumatic lesions are known as myiasis (see the review by Hall 1991), which can lead to host death if untreated. As expected by its biology, *C. hominivorax* is responsible for a large economic impact in livestock production (Alexander 2006; Grisi et al. 2014), and although its effect on wildlife and human beings remains undetermined, the Screwworm fly is considerate a high-consequence pest of all its potential hosts. Either for its veterinary or medical importance, *C. hominivorax* certainly warrants its horror-title as "*Man-eater*" (as roughly translated from Latin). From an evolutionary viewpoint, the Screwworm fly represents an ideal biological model for examining the genetic background that underlies the niche specialization in blowflies. That is due to the fact that *C. hominivorax* is not only the sole obligatory ectoparasite amongst the *Cochliomyia* genus (which includes four species endemic to the Americas), but also the only one in the Neotropical region (Guimarães and Papavero, 1999; Alexander, 2006), in opposition to all its close relatives, which are mainly carrion feeders (Yusseff-Vanegas and Agnarsson 2016). Surprisingly, and in contrast to its importance, small efforts have been made to dissect the genetic basis of *C. hominivorax*'s preference towards living flesh.

Here, we hypothesized that olfaction played a critical role in the adaptive transition from a necro-saprophagous to the obligatory ectoparasitic habit in the *C. hominivorax* lineage. In insects, the sense of odors in complex environments is a delicate process mediated by several chemosensory genes expressed in porous sensilla (hairs) attached to olfactory organs, such as the antennae and maxillary palps. Inside the sensilla, odors are solubilized and transported through the inner lymph to the receptor sites within the olfactory sensory neurons (OSNs). Two distinct olfactory receptors families, named ionotropic receptors (IRs) and odorant-selective receptors (ORs), are responsible for odor identification, which initiates a cascade of neural

events leading to behavioral response (Benton 2006; Carey and Carlson 2011; Silbering et al. 2011; Bohbot and Pitts 2015). Interestingly, the proper function of all ORs is dependent on a common Olfactory Receptor Co-receptor, named Orco (Larsson et al. 2004; Benton et al. 2006; Sato et al. 2008). Therefore, it is expected that a failure to encode Orco will result in the complete silencing of the ORs family, but not the IRs. In this context, an Orco-knockout strain of *C. hominivorax* would provide us with a great system in which olfactory behaviors mediated by ORs and IRs pathways in this species can be rapidly profiled. Hence, in this study, we present the molecular characterization, evolution and expression profiling of the Orco gene in *C. hominivorax* (*ChomOrco*). We further developed the first olfactory mutant strain of the Screwworm fly by using the Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) and the associated protein 9 (Cas9) genome editing technology (shortly CRISPR/Cas9; Doudna and Charpentier, 2014). Together with the progressively availability of blowflies' genomes (Vicoso and Bachtrog 2015; Anstead et al. 2015; Andere et al. 2016), including for *C. hominivorax* (M.J. Scott, unpublished; A.C.M. Junqueira, unpublished), the well-supported phylogenetic relationships among the *core*-Calliphoridae clade (Kutty et al. 2010; Marinho et al. 2012; Junqueira et al. 2016; but see Kutty et al. 2019), and the disposal of established functional genomic tools and protocols for reverse genetics in blowflies (Concha and Scott 2009; Li et al. 2013; Paulo et al. 2019), we believe that the methods and data described here will provide an exceptional opportunity to mechanistically investigate the genetic basis of adaptive ecological transitions in the Calliphoridae family.

METHODS

Screwworm

The wildtype (wt) strain J06 of *Cochliomyia hominivorax* was used in this study. The strain is routinely reared in the ARS laboratory located inside the COPEG biosecurity plant in Panamá under conditions previously described by Concha et al. (2016). Samples of *Cochliomyia macellaria* and *Chrysomya megacephala* were obtained from the Laboratory of Genetics and Animal Evolution, at the Department of Genetics,

Evolution, Microbiology and Imunology of the State University of Campinas (UNICAMP).

Isolation of full *ChomOrco* sequence

RNA and cDNA. Specimens were collected from colonies, rinsed with 0.1% DEPC-treated water and immediately homogenized in TRIzol reagent (Invitrogen). Tissue-specific samples were dissected from frozen 3d-old adult flies fasted for 12 h. Samples were kept at -80° and total RNA isolated according to manufacturer's instructions with minor modifications. Purity and integrity of the extractions were accessed in a NanoDrop 2000 spectrophotometer (Thermo Scientific) and by denaturing 1% agarose-formaldehyde gel electrophoresis. Quantifications were made in a Qubit 2.0 fluorometer (Invitrogen). All extractions were DNase-treated in a 20 µl reaction containing 4 U of TURBO DNase (Invitrogen), 20 U of RiboLock RNase Inhibitor (Thermo Scientific), 1x Reaction Buffer and 10 µg of total RNA. Digestions were performed at 37° for 30 min and stopped by the addition of 15 mM of EDTA (pH 8.0) followed by an incubation at 75° for 10 min. First-strand cDNAs were synthetized from 2 µg of DNase-treated RNA using the SuperScript II (Invitrogen) protocol with the Oligo$(dT)_{12-18}$ primer (Invitrogen). Reverse transcriptions were performed at 42° for 1 h and terminated at 70° for 15 min. All cDNAs were quantify as before and stored at -20° prior to further manipulation.

RACE. The specific primers Orco-F1 and Orco-R1 were first designed based on high conserved Orco nucleotide motifs in calliphorids. First strand cDNA was used as template for the amplification of a 660 bp region of the *ChomOrco* transcript in a 50 µl PCR reaction containing 0.2 µM of each primer, 80 µM of dNTPs, 10% BSA (5 mg/ml), 1x *Taq* buffer supplied with 1.5 mM of $MgCl_2$ and 1.25 U of recombinant *Taq* DNA polymerase (Invitrogen). Amplifications were carried out in the following conditions: 95° for 3 min, 35 cycles of 95° for 30 sec, 60° for 45 sec and 72° for 60 sec, and a final extension step at 72° for 5 min. PCR products were resolved in a 1.5% agarose gel stained with GelRed and purified using the QIAquick PCR Purification Kit (Qiagen). Fragments were directly TA cloned into a pGEM-T Easy Vector (Promega) and selected clones were sequenced in a 3730xl DNA Analyzer (Applied Biosystems) using the

universal primers M13-Forward and M13-Reverse. The resulting sequences were used to design specific inner primers. Classic rapid amplification of cDNA ends (Classic-RACE) was performed as described by (Scotto–Lavino et al. 2006, 2007) with minor modifications. For 3'-UTR isolation, a cDNA was prior synthetized using the RT-Q_t primer and used as template in a first RT-PCR with the universal primer RT-Q_0 and the primer Orco-F1, in the same conditions described before. The resulting amplicon was 20-fold diluted in 1x TE (10 mM Tris, 1 mM EDTA, pH 8.0) and used as template for a nested RT-PCR with the inner primers RT-Q_i and Orco-F2. The isolated 1.6 Kb amplicon was purified, TA cloned and sequenced. Overlapping fragments were then assembled with CAP3 (Huang and Madan 1999). For 5'-UTR isolation, a cDNA was prior synthetized using the Orco-R1 primer, purified by organic extraction with Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v, pH 6.7), precipitated with sodium acetate (3 M, pH 5.2) and absolute ethanol, and resuspended in 1x TE buffer. An artificial poly(A) tail was attached to the 5'-end of the cleaned cDNA with 30 U of terminal transferase (TdT, Invitrogen) and 200 µM of dATP. The cDNA was denatured at 94° for 3 min, the tail synthetized at 37° for 30 min, and the TdT enzyme inactivated at 70° for 10 min, followed by a second ethanol precipitation. The tailed-cDNA was used as template in a first RT-PCR with the universal primers RT-Q_t and RT-Q_0, and the primer Orco-R1. Amplification was made as described above after a round of initial denaturation at 95° for 5 min, pre-amplification at 48° for 2 min and pre-extention at 72° for 15 min. The resulting amplicon was diluted as before and used as template for a nested RT-PCR with the inner primers RT-Q_i and Orco-R2. The resulting 1.5 Kb amplicon was cloned, sequenced, and assembled as before. The isolated coding sequence (CDS) was translated using the ExPASy translate tool (Gattiker et al. 2003), and checked for the presence of the *7tm6 Odorant Receptor* conserved domain (pfam02949) by Domain Enhanced Lookup Time Accelerated (DELTA)-BLASTp analysis.

Genomic region. RACE-isolated *ChomOrco* sequence was used to screen the *C. hominivorax* draft genome scaffolds for the presence of intronic regions using tBLASTn-mapping analysis. Exonerate (Slater and Birney 2005) was then used to predict exon-intron boundaries on the putative genomic region of *ChomOrco*. The resultant genomic map was then screened to design specific primers for long-range

PCRs (long-PCR), which were carried out to fill sequence gaps and validate the predicted genomic region using the Long PCR Product Sequencing (LoPPs) method (Emonet et al. 2006). Briefly, total DNA was extracted from frozen adult flies using the DNeasy Blood and Tissue Kit (Qiagen). Long-PCRs were performed in a 50 µl reaction, containing 0.2 µM of each specific primer, 20 µM of dNTPs, 1x Reaction Buffer B, 1 U of Elongase enzyme mix (Invitrogen) and 50 ng of template DNA. Amplifications conditions consisted of an initial denaturation at 94° for 60 sec, 40 cycles of 94° for 30 sec, annealing at 50° for 30 sec and elongation at 68° for 8 min, followed by a final elongation step of 68° for 16 min. Long amplicons were resolved in a 0.8% agarose gel post-stained with GelRed, and diluted to 10 ng/µl in 100 µl. DNA was sheared by sonication in a Bioruptor plus UCD-300 (Diagenode) through 2 cycles of 15 sec ON and 90 sec OFF, in a 4° waterbath. Fragments were then purified with the PureLink PCR purification kit (Invitrogen) using the Selective Buffer B3, and 500 ng of purified product repaired in a reaction containing 1x T4 DNA polymerase Buffer, 1 mM dNTPs mix, 0.2 mg/ml of BSA and 15 U of T4 DNA polymerase (Invitrogen) for a final volume of 12 µl. Repair reactions were incubated at 12° for 15 min followed by the addition of 10 U of Klenow fragment (Thermo Scientific) and a second incubation at 37° for 30 min and 72° for 20 min. Resulting blunt-end fragments were phosphorylated in a 100 µl reaction containing 1x T4 DNA ligase buffer supplemented with 1 mM of ATP and 25 U of T4 Kinase (Invitrogen). Phosphorylation was performed at 37° for 30 min, and stopped at 75° for 20 min. Ready-to-clone fragments were precipitated with NaOAc (3 M, pH 5.2) and absolute EtOH at -80° for 3 h, washed once with 70% EtOH, and resuspended in 1x TE. Sub-libraries were constructed by incubating 100 ng of the prepared fragments with 100 ng of linearized pBlueScript II SK (+) vector (Agilent) in a 20 µl reaction containing 1x T4 Rapid Ligation buffer and 3 U of T4 DNA ligase (Promega). Ligations were performed overnight at 16° and transformations were made overnight at 37° in Dh10β competent cells. Sub-products were investigated via PCR using the universal primers M13-Forward and M13-Reverse. Clones hosting ~ 900 bp inserts were Sanger sequenced and assembled using CAP3. Previously isolated *ChomOrco* CDS sequence was used to anchor the long reads during the assembling process. Table S1 contains the specifications for all primers used during the isolation of *ChomOrco* sequence.

Evolutionary analysis

Diptera Orco sequences were codon-aligned using Muscle algorithm implemented in TranslatorX tool (Abascal et al. 2010). Neighbor-Joining (NJ) method was used to estimate uncorrected evolutionary distances (*p*-distance) between pairs of aligned sequences in MEGA7 (Kumar et al. 2016). For tree reconstructions, gaps were eliminated in pairwise comparisons and node supports were measured by 1000 bootstrap replicates. Maximum-likelihood (ML) trees were inferred in RAxML (Stamatakis 2014) using the PROTGAMMA model and JTT substitution matrix for the amino acid alignment, and the GTRGAMMA model for the codon-based nucleotide alignment. Node supports were accessed by 500 non-parametric bootstrap replications. Mosquito clade (*Aedes aegypti* + *Anopheles gambiae*) was defined as outgroup. Examinations for adaptive evolution were performed using CodeML (Yang 2007). Normalized non-synonymous (d_N) to synonymous (d_S) substitution rates (ω) were estimate using branch-site models (Zhang et al. 2005) to detect events of episodic selection on amino acid sites at specific lineages (referred as the foreground branches). Likelihood ratio tests (LRTs) were performed between the alternative model bsA (positive selection) and the null model bsA1 (neutral). Significances of LRT results were determined by χ^2 testes, and Bayes Empirical Bayes (BEB; Yang et al. 2005) analysis was used to infer amino acid sites under selection regime (≥ 0.95). Consensus locations of TM domains within *ChomOrco* were predicted by TOPCONS (Peters et al. 2015), and significantly characterized sites were mapped onto the predicted protein topology modeled in Protter (Wollscheid et al. 2013).

Electron Microscopy

Adult 2d-old females were fresh collected, fixed in absolute ethanol and washed in a dilution series of amyl acetate. Specimens were dried on a Denton DCP-1 Critical Point Drying apparatus (Denton Vaccum Inc.), mounted on aluminum stubs and sputter-coated with gold palladium alloy in a Hammer VI-A (Anatech) system. Screwworm' heads were observed in a Zeiss EVO 40 scanning electron microscope (Carl Zeiss AG) at the Electron and Confocal Microscopy Laboratory, Smithsonian Tropical Research Institute (Earl S. Tupper Center).

Expression

Quantitative evaluation of *ChomOrco* expression was investigated by Quantitative Real-Time PCR (qPCR). First-strand cDNAs (equivalent to the amount synthesized from 50 ng of treated RNA) were used as template in 12.5 µl amplification reactions containing 6.25 µl of SYBR Green PCR Master Mix (Applied Biosystems), and 0.4 µM of each Orco-F2 and Orco-R2 primer (Table S1). Runs were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the following protocol: hold at 50°C for 2 min and 95°C for 10 min (polymerase activation), followed by 40 cycles of 95°C for 15 sec (denaturation) and 62°C for 60 sec (Anneal/Extend). Melting curves were assessed to ensure unique product amplification. Data were analyzed with automatic threshold and baseline settings. The expression level of *ChomOrco* from each development stage was normalized to *Gapdh* (as previously evaluated by Cardoso et al. 2014) using the $2^{-\Delta Ct}$ method and presented as fold-change relative to the third instar larvae development stage using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). Values of cycle thresholds above 35 were considered undetected. Independent biological (n = 3) and technical (n = 3) replicates were carried out for all experiments, in addition to no template controls (NTC). The amplification efficiency was prior evaluated by standard curve method as shown in Figure S1. Semi-quantitative amplifications for *ChomOrco* and *CmegOrco* were made in replicates (n = 3) by Reverse Transcription PCR (RT-PCR). First strand cDNAs were used as templates in 25 µl PCR amplifications as described above. *Gapdh* was amplified from each sample as an internal control.

Intrapuparial Development

Pupas were collected 3, 6 and 8 days after pupation and fixed in Carnoy solution (ETOH: Acetic Acid: Chloroform, 6:3:1 (v/v)) for 48 h. Samples were transferred to 5% formic acid for an additional 48 h, and then kept in 70% ethanol. Specimens were dissected, and then photographed with a Nikon P-FLAP 2 stereomicroscope system.

CRISPR/Cas9

CRISPR experiments were performed using our previously described protocols (Paulo et al. 2019). Briefly, single guide RNAs (sgRNAs) were designed by examining

ChomOrco exons for the presence of protospacer-adjacent motifs (PAMs; NGG-3') using the standalone version of CRISPOR (Concordet and Haeussler 2018) in the context of *C. hominivorax* draft genome assembly (M.J.Scott, unpublished; Table S1). Syntheses of sgRNAs were performed as described by Bassett and Liu (2014), while recombinant Cas9 protein was obtained commercially (PNA Bio). Ribonucleoprotein complexes (RNPs) were pre-assembled by incubating Cas9 protein (500 ng/µl) with sgRNA (200 ng/µl) in a Sodium Phosphate Buffer (supplied with 300 mM of KCl) at 37° for 10 min. The high concentration of Cas9 was used as we previously observed to induce higher frequency of germline mutations in *C. hominivorax*. For dual-targeting experiments, RNPs were pre-assembled for each sgRNA separately and then 1:1 mixed. The plasmid pB[Lchsp83-ZsGreen] (described in Concha et al. 2011) was add (300 ng/µl) to the final injection cocktail, which was maintained on ice during the experiments. Microinjections were performed at the posterior end of pre-blastoderm embryos of the wt strain J06, and eclosing G_0 larvae transiently expressing the *ZsGreen* marker were selected and raised to adulthood.

Illumina

Genomic DNA of G_0 flies marked with *ZsGreen* fluorescence (e.g., successfully microinjected embryos) was extracted using the DNAreleasy Advance Direct Lysis Kit (Bulldog bio). Internal primers were used in a step-up PCR to amplify the Cas9-targeted regions on exons 1 and 3 of *ChomOrco*. Amplicons were gel extracted and used as template in a second PCR to incorporate Illumina adaptors and barcodes. Libraries were sequenced on an Illumina HiSeq and resulting paired-end raw reads cleaned using Trimmomatic (Bolger et al. 2014). Surviving reads were connected using COPE (Liu et al. 2012), aligned to the wt reference sequence and examined for the presence of indels using the CRISPResso (Pinello et al. 2016) and CrispRVariants (Lindsay et al. 2016) pipelines.

Mutant strain

Genomic DNA was extracted from a single midleg of microinjected adult flies (at G_0) using our custom non-lethal method (Figure S2), and used as template for PCR amplifications spanning the Cas9-targeted site at *ChomOrco* exon 1. Amplifications

were submitted to T7EN1 cleavage assays as previously described (Paulo et al. 2019), and ten mosaic males were individually backcrossed to wt females (1 Cas9 G_0 ♂ x 4 wt ♀). On average, eight G_1 adult males from each crossing were randomly selected, kept separately and genotyped as before. Nine heterozygous male flies (one per generated line, see Results) were selected and individually backcrossed to wt females for a second time. Simultaneously, Sanger sequencing and CRISP-ID (Dehairs et al. 2016) analysis were conducted for these selected flies. Adult G_2 males and females hosting a -16 bp deletion in the *ChomOrco* exon 1 were identified by T7EN1 and heterozygous siblings (e.g., from the same backcross) let to interbreed freely in cages. Homozygous mutant siblings at G_3 were identified by *in vitro* Cas9-assay, using the Guide-it Genotype Confirmation Kit (Takara), and inbred to establish the homozygous mutant line at G_4.

Immunostaining

Antennae were dissected from female' flies and frozen in O.C.T. Compound (Sakura Tissue-Tek) at -20° directly without fixation. Cryosections were made in a Leica CM1850 at a thickness of 18 µm, and thaw-mounted on gelatin-coated microscope slides. Sections were air-dried at room temperature for 10 min, submerged in phosphate-buffered saline with 0.05% Azide (PBS) for 5 min, and blocked in PBS with 0.2% Triton X and 3% Bovine Serum Albumin (PBSTB) for 1 h. Sections were incubated with anti-OR83b (Peptide for IC3: HWYDGSEEAKT, described by Benton *et al.* 2006) rabbit polyclonal antibody at 1:100 dilution in PBSTB overnight at 4° in a humid chamber. Slides were washed for a total of 15 min in PBST (5 min / wash), and incubated at room temperature for 1 h with donkey anti-rabbit Alexa 488 secondary antibody (Life Technologies) diluted 1:500 in PBSTB. Slides were washed as before, with the addition of DAPI during the last wash, mounted using Vectashield medium (Vector Labs), and sealed with nail polish. Stained antennae sections were imaged on a Leica TCS SP5 II confocal microscopy at the Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP). The wt and Orco mutant samples were mounted in the same slides and imaged using the same settings. Control slides were made without the addition of primary or secondary antibody, and no signal was detected (Figure S3).

Behavior

For the honey bioassays, unmated adult flies of 3-to-4-day-old were fasted for 12 h with access to water, transferred without anaesthesia to a test metal wire cages (BioQuip Inc., model 1450B), and left for 15 min before the trials to acclimate. Groups of 15 - 25 flies (males and females, ratio about 1:1) were tested in each trial, only once in the same day. Odorant bait consisted in 400 mg of natural honey (handcrafted at Holambra-SP, Brazil) applied on a 25 mm diameter Whatman filter paper (GE Healthcare) 20 min prior to trials, while the control bait contained the same weight of glycerol (Sigma-Aldrich). Two-choice trap bioassays were performed under controlled conditions (25 ± 2°, 60 ± 5% RH, 12:12 L:D) during light time, as Screwworm adults display a foraging behavior mainly during daytime (Thomas 1993). Testing cages were positioned right below fluorescent lights and side-enfold with white paper sheets to provide a homogeneous light distribution from above, and thus minimizing bias towards one side of the cage. For oviposition bioassays, groups of 10 - 20 fed and presumable matted adult females of 6-to-8-day-old were tested in each trial. The odorant bait contained 10 ml of 25% warmed waste larval rearing media prepared as described by Chaudhury et al. (2012), while warmed distilled water was used as control. Bioassays were performed as described above but in complete darkness. Control trials were made in order to ensure that flies did not prefer one side of the cage upon the other (Figure S4). Ablated "*antennaless*" flies (e.g., adults that had at least the 3rd antennae segment physically removed the day before trials) were also tested in order to ensure that choices were olfaction-guided. Non-lethal traps were handmade from plastic soft drink bottles, and contained two parts: the "*Collection Chamber*", formed from the upper cone-shaped funnel part of the bottle (60 mm height and Ø). Two entries (35 mm apart from each other) were made in this part by cutting the plastic in an "X" shape (5 mm Ø opening). The resulting triangular portions were folded-in and used to support a cut proximal end of a 1 mL pipette tip, which allows flies to enter the traps but prevents them to scape; the "*Bait Chamber*" was made from the bottom part of the bottle (30 mm height). The two parts connect by pushing the collection chamber into the bait chamber. Top of traps were closed with fabric screen to allow air ventilation, and baits were placed in the center of plastic

dishes in the inner bottom of the bait chamber. Traps were alternatively placed in opposite sides of the testing cages, and their positions altered after every trial. Trap captures were scored after 6 h (honey) or 90 min (oviposition), and attraction index (AI) was calculated as:

$$AI = (n_{odor} - n_{control}) / n_{total}$$

where n_{odor} is the number of flies captured in the odorant trap, $n_{control}$ the number of flies present in the control trap, and n_{total} the total of flies in the test cage. AI ranges from −1 (complete avoidance) to 1 (complete attraction), whereas zero characterizes a neutral or non-detected odorant. Significant deviations of AI were tested with the Wilcoxon rank sum test.

Data availability

Final *Cochliomyia hominivorax* Orco (*ChomOrco*) and *Cochliomyia macellaria* Orco (*CmacOrco*) sequences will be submitted shortly to GenBank. Additional tables and figures are available as Supplemental Material at the end of this Chapter. A C. *hominivorax* heterozygous strain for the -16 bp Orco mutation (named Orco16Ko) is being maintained at the ARS laboratory inside the biosecurity plant at COPEG, Panamá-PA.

RESULTS

Screwworm Orco gene is highly conserved within Diptera order

Classic-RACE was used to isolate the full-length Orco transcript sequence of *C. hominivorax*, which is represented by 1,437 bases pair (bp) encoding a 478 amino-acid (aa) peptide sequence. The *ChomOrco* transcript shares 73 and 92 % of nucleotide identity with Orco sequences from *Drosophila melanogaster* and the closely related Oriental latrine blowfly *Chrysomya megacephala*, respectively. As expected between Orco orthologous (Vosshall and Hansson 2011), the *ChomOrco* coding sequence exhibit an extremely high amino acid identity with all dipterans investigated (mean ± SD: 92 ± 5.5 %, Table S2). Based on its transcript sequence, we next used a combination of genome-wide prediction and long-range PCR sequencing methods to isolate the complete 12,870 bp genomic region corresponding to the Orco gene in Screwworm. Genomic organization of *ChomOrco* is characterized by the presence of 7 exons, highly conserved among dipterans, separated by 6 introns (Figure 1A and B). Differently from

D. *melanogaster*, *ChomOrco* exon 2 is subdivided into two parts (referenced here as E2a and E2b) separated by a specific 74 bp intronic region (named I1b; Figure 1A). Interesting, this seems to be an organization shared by all Calyptratae species (data not shown). Membrane protein topology predictions indicated that the Screwworm Orco contains seven transmembrane domains (TM), an inverted terminal membrane topology (N_{in}-C_{out}) and a conserved tyrosine residue at TM7 (marked "Y" in Figure 1A and B), all signatures of this atypical OR co-receptor (Benton et al. 2006; Nakagawa et al. 2012). Most conserved residues were found at TM6 and TM7, which is thought to be a region were the Odorant Receptors (ORs) partially interact with Orco. As evidenced in other calyptrates (see Olafson (2013) for an example), *ChomOrco* is eight amino acids (^{309}NGGGGNGL^{316}) shorter than *Drosophila* at the intracellular loop 2 (IC2), connecting TM4 and TM5, a region believed to be important for intracellular transport (Benton et al. 2006). The long IC2, in comparison with the conventional ORs, is another Orco feature (Wicher et al. 2008; Sato et al. 2008) and this region appears to be a commonplace for Orco length variations, at least in dipteran species (Figure 1B), suggesting that a more relaxed selective pressures are acting on this motif when compared to other regions of the protein.

Gene tree inferences recovered *ChomOrco* in the Calliphoridae (blowflies) clade sharing a common node with Orco sequences from muscids, and this node forming a sister-clade with drosophilid or tephritid's sequences (Figure 2A), consistently reflecting the phylogenetic relationships among the Schizophora clade (Wiegmann et al. 2011; Junqueira et al. 2016). We next interrogate the branch leading to *C*. *hominivorax* species for site-specific signatures of episodic diversification, as positive selection pressures are likely to affect only a few sites within a specific lineage.

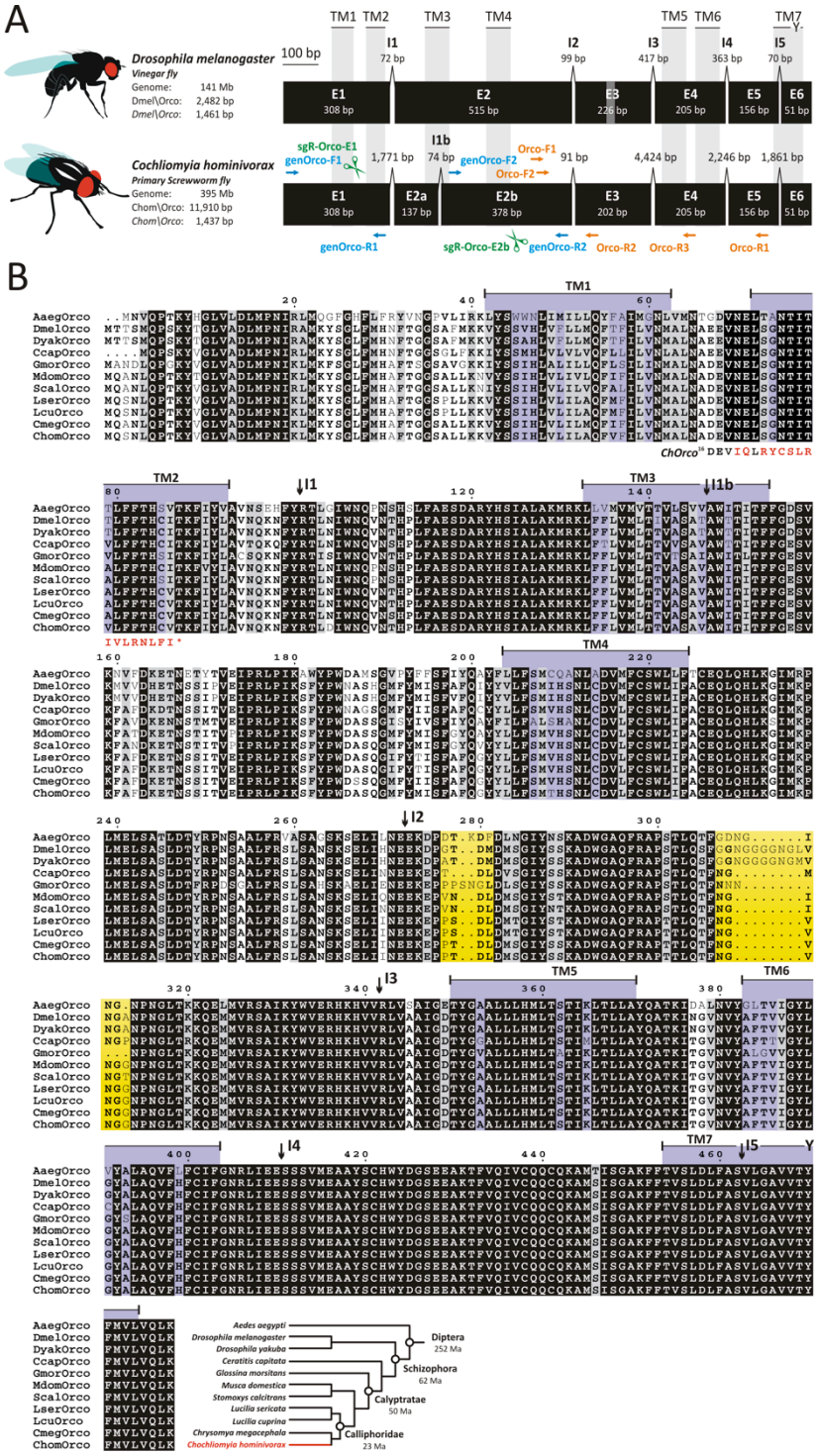

Figure 1 Experimental design and molecular characterization of Orco gene from *Cochliomyia hominivorax*. **(A)** Comparison between Orco gene organization in the vinegar fly, *D. melanogaster* (*DmelOrco*) and the Screwworm fly, *C. hominivorax* (*ChomOrco*), in the context of their genome sequences. Exons are represented by numbered black boxes (E1-E6) and introns as connecting lines (I1-I5). Positions of the transmembrane domains (TM), the conserved tyrosine residue at TM7 (Y) and nucleotide length variation at E3 are also being represented. Location of the primers used for *ChomOrco* isolation, expression analysis, and molecular genotyping are shown by arrows, while Cas9 RNPs targeted sites at E1 and E2b are indicated by scissors (all primer specification can be found in Table S1). **(B)** Multiple sequence alignment of Orco within Diptera. Black arrows indicate locations of the six identified introns in *ChomOrco* genomic region. Purple boxes indicate the position of the seven TMs, while nucleotide length variations at E3 region are highlighted in yellow boxes. Modifications introduced by CRISPR\Cas9 in *ChomOrco* (see Results and Discussion below) are shown below the alignment. The evolutionary relationships among species analyzed are given at the end of the alignment, according to Junqueira et al. (2016). Species abbreviations and accession numbers are as follows: Aaeg (*Aedes aegypti*, NM_001358471.1); Dmel (*Drosophila melanogaster*, AY567998.1); Dyak (*Drosophila yakuba*, XP_002096053); Ccap (*Ceratitis capitata*, XM_012300753.1); Gmor (*Glossina morsitans*, available by Obiere et al. 2014); Mdom (*Musca domestica*, JQ365179.1); Scal (*Stomoxys calcitrans*, EU622914.1); Lser (*Lucilia sericata*, HQ315862.2); Lcup (*Lucilia cuprina*, XM_023445888.1); Cmeg (*Chrysomya megacephala*, HQ315861.2), and; Chom (*Chochliomyia hominivorax*, this study).

Results revealed that the selective pressures in the Screwworm lineage do not differ from the background tree, presenting signals of a very strong purifying selection

(ω_{K0} = 0.025) in the majority of the corresponding amino acid sites (97.3 %), while remain sites showed evidence of relaxed constraint ($\omega_{K1} \approx 1$; Figure 2A and B). Only a single peptide (^{211}threonine, located inside TM4) exhibited some signal of positive selection in the *Cochliomyia* clade ($\omega_{frg} \geq 1$, while $\omega_{bkg} < 1$), although not statistically significant (BEB score: 0.92). Thus, it's more likely that this site is rather experiencing a relaxation of selective constraints in this clade. The same evolutionary signatures were obtained when testing the branch leading to *Drosophila suzukii* lineage (Figure 2A), revealing that Orco sequence conservation might reflect its indispensable role in olfaction across taxa.

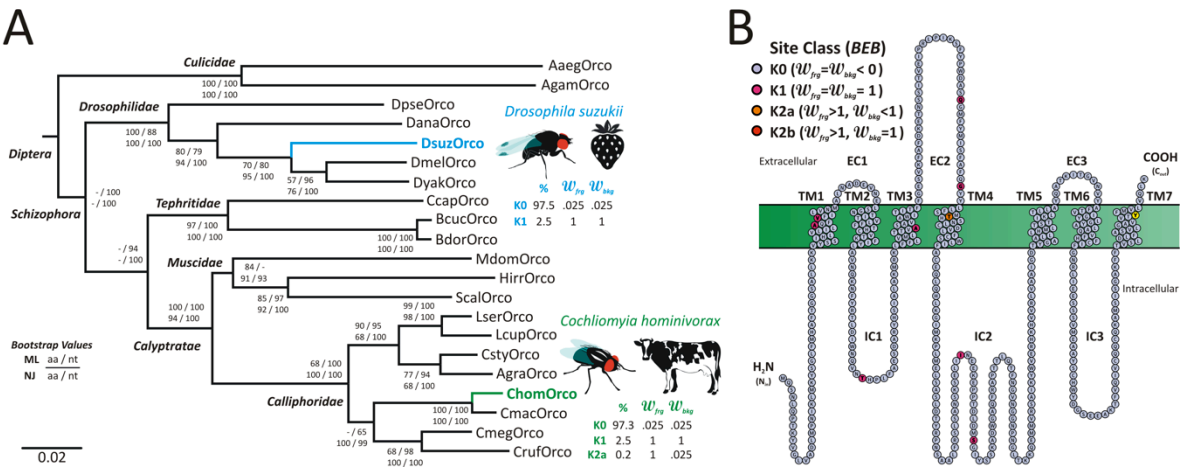

Figure 2 Evolutionary conservation of *ChomOrco*. (A) Consensus phylogenetic relationships of dipteran Orco sequences as given by Maximum Likelihood (ML) and Neighbor-joining (NJ) analysis. Bootstrap support values recovered from each analysis are shown close their respective nodes. Normalized non-synonymous (d_N) to synonymous (d_S) substitution rates (ω) were estimated using branch-site models (Zhang et al. 2005) to test the branches leading to *C. hominivorax* (*ChomOrco*, in green) and *Drosophila suzukii* (*DsuzOrco*, in blue) species for events of episodic selection. The number of sites estimated to be evolving under purifying (site class K0), relaxed (K1) and positive (K2a and K2b) regime are shown for each foreground branch (ω_{frg}) tested in relation to the rest of the tree (background branch; ω_{bkg}). Abbreviations and accession numbers (additionally to Figure 1B) are as follows: Agam (*Anopheles gambiae*, Q7QCC7.3); Dpse (*Drosophila pseudoobscura*, XM_001359327.3); Dana (*Drosophila ananassae*, XP_001953343); Dsuz (*Drosophila suzukii*, NM_001328601.1); Bcuc (*Bactrocera cucurbina*, HM745934.1); Bdor (*Bactrocera dorsalis*, EU621792.1); Hirr (*Haematobia irritans*, EU622915.1); Csty (*Calliphora stygia*, KJ702047.1); Agra (*Aldrichina graham*, HQ190955.1); Cmac (*Cochliomyia macellaria*, this study), and; Cruf (*Chrysomya rufifacies*, JQ365176.1). **(B)** Significant characterized sites by Bayes Empirical Bayes (BEB; Yang et al. 2005) analysis mapped onto the predicted protein topology of *ChomOrco* as modeled by Protter (Wollscheid et al. 2013).

Expression of Orco is conserved during blowflies' development and is broadly detected in Screwworm olfactory-related tissues

We next investigated the relative expression of Orco during Screwworm development by Quantitative Real-Time PCR (qPCR). Results revealed that Orco transcription takes place during the final stages of embryo development (E12h), and it's maintained at low levels during the early immature stages of *C. hominivorax* (L1 until L3 in Figure 3A). Following the last larval stage (L3), the pupae stage is characterized by a gradual increase in Orco abundance over time (P3d to P8d in Figure 3A), reaching its highest level during the pre-imago phase (P8d). Upon dissection, it was possible to verify that late developed pupae lie on the pharate intra-puparian stage, characterized by the full development of the adult form and its main olfactory appendages (Figure 3B). Indeed, Orco transcription reaches the highest level in the adult stage (AD in Figure 3A). These morphological and transcriptional observations corroborate with the idea that Orco expression follows the transition from a primordial larvae sensory system to an adult more complex one (Larsson et al. 2004), also suggesting that olfaction plays a major role in the adult life stage of the Screwworm fly. Asking if this developmental pattern would be evolutionary conserved, Orco transcription was also investigated during the development of the blowfly *C. megacephala.* Semi-quantitative Reverse Transcription PCR (RT-PCR) assays showed that these species share a very similar expression pattern of Orco (Figure 3C), suggesting that selective pressures might be likewise acting on the expression control of this gene in addition to maintaining its sequence and structure (as shown in Figure 1B).

In insects, Orco is expressed in nearly all of the olfactory sensory neurons (OSNs; Larsson et al. 2004). Therefore, it was expected *ChomOrco* to be mainly detected in olfactory-related tissues of *C. hominivorax* adults. Semi-quantitative amplification assays revealed that *ChomOrco* is robustly expressed in the main olfactory appendages of both sex of the Screwworm fly; the antennae and maxillary palps (Figure 3D). Screwworm' antennae are subdivided into three segments, the scape, pedicel and funiculus, the former hosting a thin and plumose arista (Figure 3E; panels *a* and *b*). Scanning Electron Microscopy (SEM) of females' head reveals that *C. hominivorax* funiculus is predominantly adorned by three classes of sensilla: coelonic, tricoide and basiconic (Figure 3E; panel *c*). A close view at the proximal portion of this

segment exposes a number of other two morphotypes of coeloconic sensilla, named *grooved* and *clavate* (see Fernandes et al. 2004 for detailed information), lying in deep bristle pits and surrounded by microtrichias (Figure 3E; panels *d* to *f*). These subclasses of sensilla are characterized by the presence of grooves and multiple wall-pores, thus designating an olfactory function, which is correlated to the high levels of Orco transcripts in this appendage. Apart from classic olfactory organs, a considerable Orco transcription was detected in legs and - *mainly* - abdomen of both male and female flies (Figure 3D), implicating that these appendages also have chemoperception roles in *C. hominivorax*. Interesting, Orco transcription was also detected in the ovipositor, indicating that females might use this appendage to recognize chemical cues on short-distance after host finding, presumably cooperating to elicit the egg-laying behavior.

CRISPR/Cas9 efficiently introduced somatic and inheritable mutations in the Orco locus of *C. hominivorax*

The evolutionary conservation of Orco among dipterans implies a functional one, suggesting that *ChomOrco* is crucial for OR-mediated olfactory perception in *C. hominivorax*. We tested this assumption by functionally knocking out Orco in Screwworm using CRISPR/Cas9 genome editing (Doudna and Charpentier 2014), as successful protocols to obtain loss-of-function mutants of blowflies were recently made available by our group (Paulo et al. 2019). Single guide RNAs (sgRNAs) efficiency was found to vary considerably in blowflies, thus we designed and tested *in vivo* two different sgRNAs targeting exons 1 (sgR-Orco-E1) and 2b (sgR-Orco-E2b) of the Screwworm Orco gene (Figure 4A). Microinjections were carried out with the addition of the ZsGreen fluorescent marker to the final injection cocktail, as flies carrying mutations at *ChomOrco* loci were expected to lack distinguishable visual traits from their wt counterparts.

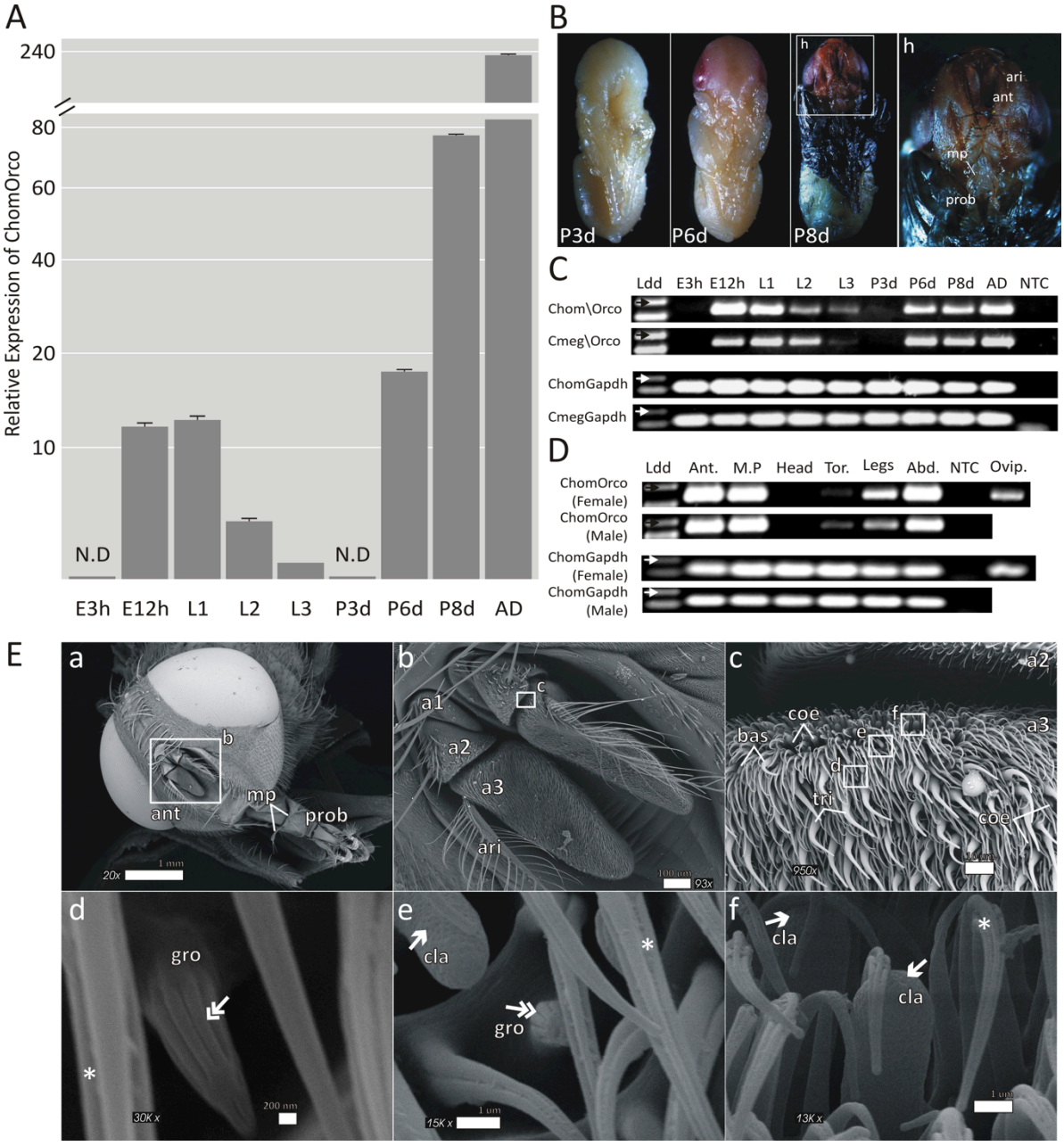

Figure 3 Developmental and tissue expression profile of Orco in *C. hominivorax*. (A) Relative expression of Orco during the Screwworm development by quantitative real time PCR (qPCR) method. Measures are given by the quantitation of *ChomOrco* threshold cycle values normalized to GAPDH and relative to the third larvae instar. Data are represented as mean ± SD (*n* = 9). **(B)** Intrapuparial development of *C. hominivorax* showing a closer view of head structures present in the pre-imago phase (*panel h*). **(C)** Comparison between Orco expression patterns during the development of *C. hominivorax* (Chom) and *Chrysomya megacephala* (Cmeg) by semi-quantitative reverse transcription PCR (RT-PCR). **(D)** Expression of *ChomOrco* in male and female tissues. GAPDH gene was amplified for each sample as an internal control. Black and white arrows indicate the molecular height of 850 bp and 200 bp, respectively. Amplifications of 660 bp and 128 bp were expected for Orco and GPDH, respectively. Abbreviations are as follows: E3h (early embryo of 3-hour-old); E12h (late embryo of 12-hour-old); L1 (first larvae instar); L2 (second larvae instar); L3 (third larvae instar); P3d (pupae 3-day-old); P6d (pupae 6-day-old); P8d (pupae 8-day-old); AD (adult); Ant/ant (antennae); **(->)**

Figure 3 (Cont.) M.P (mouth parts); Tho (thorax); Abd (abdomen); Ovip (ovipositor); ari (arista); mp (maxillary palps); prob (proboscis); NTC (non-template control), and; Ldd (1Kb plus DNA Ladder molecular marker, Invitrogen). **(E)** The olfactory structures in *C. hominivorax* viewed under electron microscopy. (*panel a*) Females' head highlighting the main adult chemosensory structures: antennae (ant); maxillary palps (mp), and; proboscis (prob). (*panel b*) Screwworm antennae are subdivided into three segments: scape (a1); pedicel (a2), and; funiculus (a3) with the attached arista (ari). (*panel c*) A closer view on the proximal surface of the funiculus revealing a number of tricoide (tri), basiconic (bas) and two morphotypes of Coelonic (coe) sensilla surrounded by Microtrichia (*), named *grooved* (gro) and *clavate* (cla), which are adorned with multi-wall pores (single arrows) and grooves (double arrows), therefore assigning an olfactory role (*painels d to f*).

Successfully injected individuals transiently expressed the marker, facilitating the screening and selection of putative somatic mutants at G_0 (Figure 4B). Ribonucleoproteins (RNPs) were pre-assembled by mixing Cas9 protein with each sgRNA separately and injected into the posterior end of a small number (*n* ~ 100) of early syncytial Screwworm embryos. Hatching first instar larvae were inspected the next day, and the ones giving strong green fluorescence were collected, pooled and DNA extracted. Total DNA extractions were used as templates for amplifications encompassing the Cas9 targeted regions within *ChomOrco* and examined for the presence of indels by T7 endonuclease 1 assays (T7EN1) and Illumina sequencing (Figure 4C and D). The analysis revealed that besides both designed sgRNAs are active, sgR-Orco-E1 overcome sgR-Orco-E2b in its efficiency rate by at least 2-fold in average (see pie charts in Figure 4C and D). We further evaluated the possibility of inducing large deletion events between the Cas9 targeted regions by injecting RNPs multiplexed with both sgRNAs into embryos (Figure 4A). The goal was to introduce a ~ 2.2 Kb deletion at the *ChomOrco* loci, which would be easily distinguished from the wt allele by routine PCR and electrophoresis. Although some degree of mutagenesis was observed in individuals developed from dual-targeting injections, large deletions were found rare and uninheritable events (Figure S5), most likely due to the efficiency differences between tested sgRNAs.

Based on these results, new microinjections were carried out using the sgR-Orco-E1 alone (*n* = 750 embryos). Out of the 329 hatching larvae (larvae surviving rate: 44%), a total of 154 presented a strong green fluorescence (putative injection success: 47%). Fluorescent marked larvae were collected and reared until adulthood, and 28 healthy adults were obtained (adult surviving rate: 18%), including 12 males and 16 females. Only surviving G_0 males were kept to backcrossing, as a high level of sterility was expected for females developed from CRISPR/Cas9 microinjections (Paulo et al.

2019). Non-lethal DNA extractions (see Figure S2) were used as templates for T7EN1 genotyping, which revealed that all selected males hosted indels at the *ChomOrco* loci (Figure S6). Ten males were randomly selected and individually backcrossed to virgin wt females to exam their founder capacities (Figure 4E). For each crossing, another 8 males (on average) were randomly sampled and genotyped as before. Out of the ten putative founders, nine produced heterozygous G_1 offspring (transmission efficiency: 90%) at percentages ranging from 14 to 89% (Table S3), revealing Cas9-induced inheritable mutagenesis. Nine G_1 heterozygous males (one per obtained line) were selected and individually backcrossed to wt females for a second time. Simultaneously, DNA extractions were carried out for these flies, followed by PCR amplification and Sanger sequencing. Resulting chromatograms were examined using the CRISP-ID application (Dehairs et al. 2016) to access the allele variants harbored by each putative founders. In total, six mutated alleles were recovered, including three variants that disrupted the open read frame of *ChomOrco*, and thus were expected to result in a non-functional Orco protein (Figure S6). Siblings at G_2 from the line harboring a -16 bp deletion ($Orco^{16/wt}$) were selected and let to inbred. The -16 bp mutation was chosen based on the likelihood of functional consequences (Figure 1B, 2B, and S7), and also due to the possibility of a simpler PCR-based genotyping approach. The putative founder carrying a similar deletion (-19 bp) did not produce viable eggs. Homozygous mutants were identified at G_3 by *in vitro* Cas9-assay (Figure S6). Out of 96 genotyped G_3 adults, 49 (51%) were heterozygous for the mutation ($ChomOrco^{16/wt}$), 19 (20%) were wt ($ChomOrco^{wt}$), and 28 (29%) were homozygous mutants ($ChomOrco^{16}$), as expected for the 1:2:1 Mendelian inheritance ratio (X^2 = 0.4; p = 0.52; d.f = 1). Homozygous individuals were confirmed by sequencing (Figure 4F), and selected flies left to inbreed to establish a homozygous strain at G_4. The knockout phenotype of $ChomOrco^{16}$ was confirmed by immunostaining. Polyclonal primary antibody IC3 against the third intracellular loop of Orco (Benton *et al.* 2006) labeled the OSNs cell body and dendrites inside sensilla on wt flies (Figure 4G, *above*), while no Orco protein was detected in mutant flies (Figure 4G, *bellow*). As in control slides, were primary or secondary antibodies were omitted, antennae sections of Orco mutants showed no labeling, confirming that the $ChomOrco^{16}$ strain was protein null.

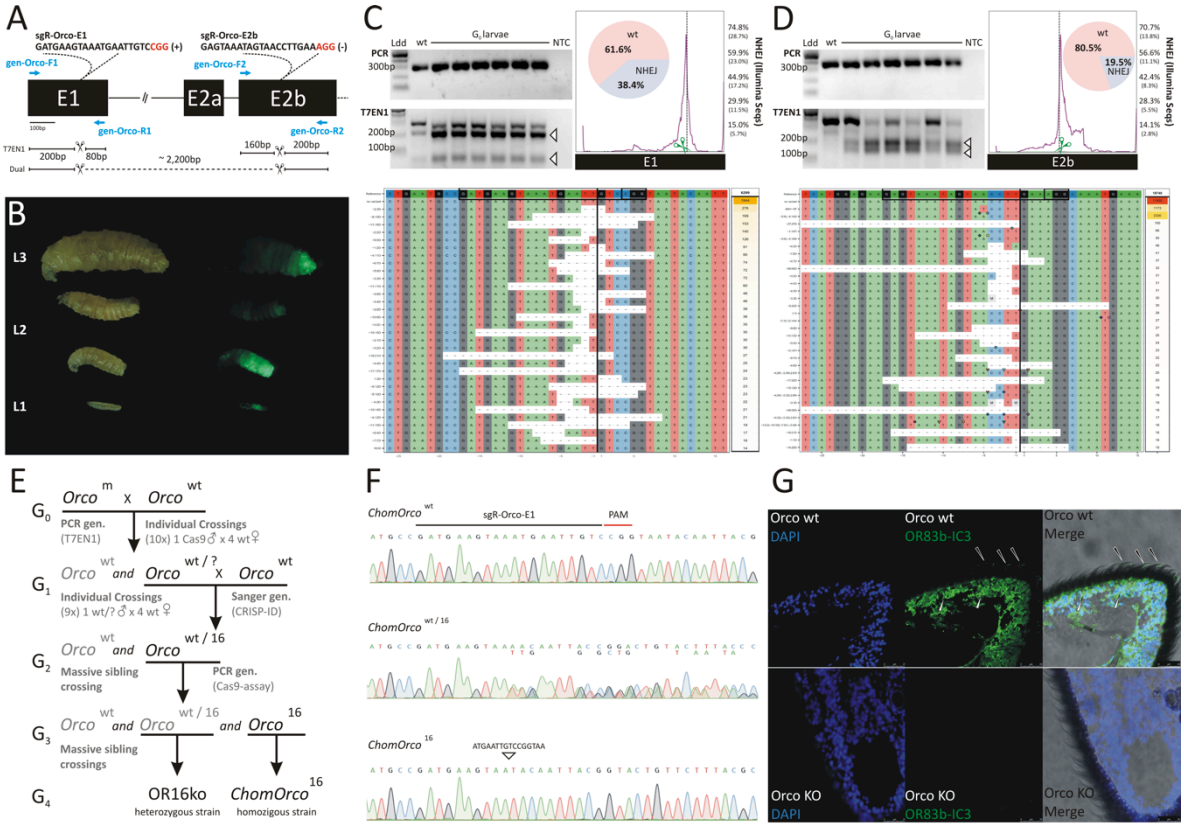

Figure 4 *In vivo* testing of sgRNAs against *ChomOrco* assisted mutant strains establishment. (A) Schematic of the 5`-proximal region of *ChomOrco* gene organization (as in Figure 1A) and CRISPR strategy used. Two sgRNAs (sgR-Orco-E1 and sgR-Orco-E2b) were designed ~ 2.2 Kb apart. Cas9 RNPs targeted sites are indicated by scissors with their respectively sgRNA (PAM motifs are shown in red). Genotyping primers are indicated as blue arrows. Band migration patterns expected for T7EN1 assays and Dual-targeting are indicated below the gene scheme. **(B)** Screwworm larvae transiently expressing the ZsGreen fluorescent marker as observed in bright (left) and fluorescent (right) stereoscope light. **(C)** *In vivo* testing of sgR-Orco-E1 targeting *ChomOrco* exon 1. A small number of fluorescent first instar larvae (*n* = 6) were collected and DNA extracted after microinjections. Extractions were used as template for PCR amplifications spanning the Cas9 targeted site followed by T7EN1 assays (leftmost upper part). Same PCR amplifications were polled and sequenced by Illumina. Sequencing reads were cleaned, assembled and mapped against the wildtype (wt) reference sequence. Allele variants introduced by CRISPR (top *n* = 35 most frequent alleles are being shown) were then examined using the CRISPResso (rightmost upper part) and CrisprVariants pipelines (lower part). **(D)** *In vivo* testing of sgR-Orco-E2b targeting *ChomOrco* exon 2b. Abbreviations are as follows: L1 (first larvae instar); L2 (second larvae instar); L3 (third larvae instar); NHEJ (non-homologous end join events); NTC (non-template control), and; Ldd (100 bp DNA Ladder molecular marker, NEB). **(E)** Crossing scheme used to stablish Orco mutant strains of the Screwworm fly. Briefly, mosaic flies (*Orco*^{m}) were obtained at G_0 after microinjections of Cas9:sgR-Orco-E1 RNPs against *ChomOrco* exon 1. Ten T7EN1 confirmed mosaic males were individually backcrossed to wt females (*Orco*^{wt}). Heterozygous individuals (*Orco*$^{wt/?}$) at G_1 were examined by Sanger sequencing followed by CRISPR-ID analysis (Dehairs et al. 2016), and siblings hosting a -16bp mutation let to inbred freely in cage at G_2. Heterozygous (*Orco*$^{wt/16}$) and homozygous (*Orco*^{16}) mutant flies at G_3 were identified by Cas9-assay, and inbred to stablish Screwworm heterozygous (OR16ko) and homozygous (*ChomOrco*^{16}) mutant strains for Orco gene. **(F)** Confirmation of heterozygous (*ChomOrco*$^{wt/16}$) and homozygous (*ChomOrco*^{16}) mutant genotypes in comparison with the wt (*ChomOrco*^{wt}) allele. The sequence of sgR-Orco-E1 is highlighting (PAM in red). Sanger sequencing chromatograms were visualized in CRISPR-ID application. Overlapping peaks beginning from the mutation site in heterozygous chromatogram are expected due to the presence of both wt and mutated alleles. **(->)**

Figure 5 (Cont.) (G) Immunostaining of antennal sections from wildtype (wt, *above*) and Orco knockout mutant (KO, *bellow*) flies, showing cell nuclei (DAPI staining in blue) and Orco protein (OR83b-IC3:Alexa-488 staining in green) localization within cell body (white arrows) and dendrites (black arrows) of Screwworm' OSNs. Mutant flies display a complete loss of Orco protein. Images are from the distal funiculus antennal segment. Scale bar is 25 µm.

Disruption of Orco impairs foraging and host-seeking behaviors in *C. hominivorax*

Homozygous Screwworm mutants for Orco do not exhibit any visible phenotype, locomotion or mating disabilities, and they are fertile. Yet, we observed a clear reduction in their overall fitness in relation to wt flies. Females of *ChomOrco*[16] take longer to lay eggs, and larvae development is arrested in addition to a higher mortality rate. Because of that, we interrogated Orco mutants for impaired OR-mediated olfactory behaviors throughout non-lethal two-choice trap bioassays (Figure 5A). That allowed us to investigated whether the disruption of the *ChomOrco* gene translates into altered olfactory-mediated responses (behavioral phenotypes) while maintaining mutant colonies under laboratory rearing conditions. The homozygous mutant strain for Orco was maintained for eight inbreeding generations before it collapsed. In this study, we focused on adult foraging and host finding behaviors, as they are central for the *C. hominivorax* ectoparasitic habit. Upon emergence, Screwworm flies spend most of its time resting and feeding on flowering vegetation (Thomas 1993). Nectar and pollen are thought to have a great impact on survivor and longevity, but most importantly in ovary maturation and reproductive success in Screwworm and other blowflies (Peterson et al. 1987; Brodie et al. 2016). Hence, we tested the implications of Orco disruption on the attractiveness of males and females to honey (odor) or glycerol (control) in trap bioassays. Honey was used as an odor cue related to floral nectar (DeGennaro et al. 2013), which it's also used as a primary source of nutrition in Screwworm rearing conditions (Figure 5B). Control trials showed that flies don't display preferences to cage sides and are not attracted to control baits (Figure S4). We found that likewise ablated "*antennaless*" flies (see methods), Orco Screwworm mutants exhibit little to no attraction to honey, while heterozygous and wt individuals shown a strong preference for this nutritional source (Figure 5C). In insects, members of the OR family are highly tuned to fruity ester-derived smells (Gomez-Diaz et al.

2018). Therefore, these results indicate that the disruption of the Orco gene most likely compromised the function of the generalist Screwworm' ORs genes.

We next asked what would be the implications of such an impaired olfactory pathway to searching for a suitable oviposition site by Screwworm females. Gravid females of *C. hominivorax* are attracted by odors released from Screwworm-infested wounds to oviposit, which are believed to be a favored oviposition site in nature (Chaudhury et al. 2002). Infested wounds release attractive odors that act as host-finding cues (Zhu et al. 2017), which can be simulated from waste larval rearing media (Chaudhury et al. 2014). Indeed, this attractant is offered to stimulate females to lay eggs in rearing conditions (Figure 5D). The attractiveness of 6-to-8-days-old mated females to spent diet (odor) or water (control) was evaluated in a second trap bioassay experiment. These experiments were conducted in a full-time darkroom, as darkness increase oviposition rates (Hammack 1991). As expected, wt and heterozygous female flies showed a strong preference to the odor source (Figure 5E), very often laying eggs inside the traps (data not shown). On the other hand, Screwwom mutants for the Orco gene have severely reduced attraction to this oviposition site, also showing no decision-making or flight orientation towards the stimuli source. The same indexes were obtained when flies had their antennae dissected. Interesting, the results not only confirmed that Screwworm host-seeking is engaged by olfaction but further indicates that the perception of host-associated chemical cues relies – *at least in part* – on the members of the OR family.

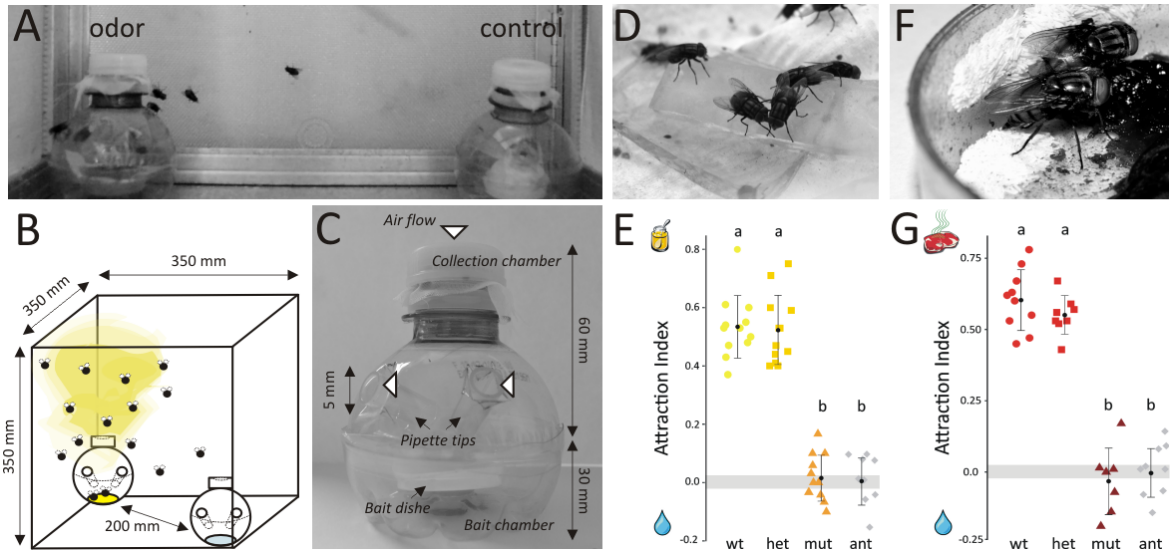

Figure 5 Orco mutants exhibit disrupted foraging and host seeking behaviors. (A) Two-choice trap bioassays were developed to examine the behavioral phenotype of Orco mutant Screwworms (see methods for details). **(B)** Test arena consisted in a square metal wire cage. Odor (yellow bait) and control (blue bait) traps were placed in opposite sides of the arena, and attraction indexes were calculated on the number of flies caught in each trap. **(C)** We developed non-lethal traps for the experiments, consisting in a collection and bait chambers. Flies were allowed to enter the traps through entries on the collection chamber handmade of pipette tips, and access the bait inside the bait chamber. Volatile release was expected to occur in air ventilation areas (white arrows), including the entrances and top of traps. **(D)** Adult Screwworms feed on a honey-based diet under rearing conditions, which odors reassemble floral nectar, a preferred nutrition source in Nature. **(E)** Orco mutants (mut; *n* = 12) lost attraction to honey odors, likewise "*antennaless"* flies (ant; *n* = 9), while wildtype (wt*; n* = 12) and heterozygous (het; *n* = 12) flies remain strongly attracted to this food source. **(F)** Gravid Screwworms are attracted by infested-wounds' odors to oviposit. Spend larval media releases the same volatiles, and is used to stimulate oviposition in rearing conditions. **(G)** Orco mutants (*n* = 7) have severely reduced attraction to the oviposition media when compared with wildtype (*n* = 10) and heterozygous (*n* = 8) flies. Indeed, mutants showed same responses as flies that had their antennae dissected (*n* = 9). In both experiments, Orco mutants showed a disrupted flight orientation towards odor source, implying that ORs are indispensable for foraging and host-seeking behavior in *C. hominivorax*. Genotypes marked with different letters are significantly different as given by Wilcoxon rank sum test. Odor traps contained honey (honey jar in the plot) or waste larval media (rotten steak), while control traps contained water or glycerol (blue drop).

DISCUSSION

The emergence of the obligatory ectoparasitism habit in blowflies has been the subject for scientific debate for over a century, calling attention to the need for studies regarding how genomic architecture is linked to adaptive behavior in this diverse group of flies. Fritz Zumpt, the medical entomologist who defined the current term "myiasis", stated in the first line of the very first chapter of his monograph: "*It is not difficult to reconstruct the evolution of myiasis*" (Zumpt 1965). By observing larvae feeding preferences, he argued that the

evolutionary route by which the obligatory ectoparasitism lifestyle arose in calliphorids derived from an ancestral saprophagous habit, in which maggots feed and breed on decaying organic matter. Some lineages, however, may have been able to colonized injured tissues of living animals, and occasionally the living ones. Primary and secondary facultative species might have emerged from this intermediate stage. In the course of blowflies' natural history, this transitional habit ultimately led to the evolution of the obligatory ectoparasitism, represented by species that are unable to develop in the absence of a living host, such as the New World Screwworm fly, *Cochliomyia hominivorax*. Zumpt's explanation confronted the earlier view held by Keilin (1915) and gained more weight after the essay published by Erzinclioglu (1989). The former additionally proposed that this parasitic lifestyle was likely to have arisen independently and multiple times during the evolution of blowflies, and might have been drastically influenced by artificial selection (animal domestication). However, it was not until thirty years later, with the advancement of molecular methods, that these hypotheses were confirmed (Stevens and Wall 1997; Stevens 2003; Wallman et al. 2005a; Junqueira et al. 2016). Timescale phylogenetic reconstructions linked the radiation of blowflies to the diversification and spread of grazing mammals during the Oligocene-Miocene border ca. 22 Ma, which might have created a favorable landscape to radiation of the Calliphoridae family. Blowfly's speciation might have been followed by intense competition for ephemeral carrion resources. Initially, this harsh condition may have favored opportunistic blowflies attracted by wounded animals, but ultimately, this struggle for food resources led to the emergence of obligatory parasites (Wall and Shearer 2008).

While those studies have shed some light on the events leading to the emergence of the obligatory ectoparasitism in blowflies, the genetic basis underlying the evolution of such a life strategy remains largely unknown. Key questions seem to rely on the understanding of genomic bridges between environment and the blowflies' biology: From signal (stimuli) to perception, and behavior. In this study, we gathered efforts to rapidly expand our current knowledge on olfactory pathways used by *C. hominivorax*, to find potential hosts for oviposition. The Screwworm genome is predicted to encode ~45 highly diverse members of the Odorant Receptor (OR) family (R.M.Gonçalves, unpublished), which are presumably involved in the majority of

odorant detection in this species, as previously found for other insects. Conveniently, the proper function of this large group of genes relies on a single highly conserved olfactory co-receptor, named Orco. Without Orco, the correct cellular localization of all conventional ORs is compromised, preventing the OR-mediated odor signal transduction (Larsson et al. 2004; Benton et al. 2006). This dependency placed Orco as the preferred gene to explore the importance of ORs in insect behavior (Mansourian et al. 2019). We found that the *C. hominivorax* Orco gene (*ChomOrco*) is highly conserved among dipterans (Figure 1), as a reflection of a strong purifying selection regime (Figure 2). The same pressures were observed for the *D. suzukii* lineage. The spotted-wing drosophila, as commonly known, is an invasive pest that relies on olfaction to find ripening fruits to oviposit, while most closely related *Drosophila* species prefer to lay eggs on rotten matter (Karageorgi et al. 2017). This adaptation for host preference can be considered analogous to the habitat preference observed in blowflies. Using genome comparative analysis, Hickner et al. (2016) revealed that while *DsuzOrco* is highly conserved, signals of adaptive evolution are detectable in few *D. suzukii's* ORs, which were later found to be differentially expressed in post-mating females (Crava et al. 2019). Therefore, it's expected that evolution might be acting on the OR gene family members, rather than on key genes such as Orco, to architect the olfactory landscape of the Screwworm fly.

The *ChomOrco* expression profile indicates that the Screwworm olfactory system experience drastic changes during development (Figure 3A). The *ChomOrco* is detectable during the last hours of embryonic development, and similar expression levels are found in first instar larvae. Screwworm females lay their eggs on dry borders of hosts' wounds and orifeces, thus hatching larvae might use olfactory clues to find suitable substrate to feed. The relatively low expression of Orco during early larval stages might be also related to their morphologically simpler chemosensory system thought to harbor a smaller number of Odorant Receptor Neurons (ORNs) in comparison with adult flies (as demonstrated by Stocker 2008). The lowest Orco expression level is observed for the third instar larvae. Differently from the previous two instars, which are considerate the "*foraging*" phase of early Screwworm development, the last larval instar enters in the so-called "*wandering*" phase, when they no longer feed and start a host exodus to pupate. It is unclear however whether

the slope in Orco expression contributes to this behavior or it is just the consequence of the pupate-signaling in *C. hominivorax*, but this pattern appears to be evolutionary conserved, as also observed in *Lucilia sericata* (Wang et al. 2012) and *C. megacephala* (Figure 3C) species. Interesting, we found that *ChomOrco* expression is correlated to changes in peripheral olfactory morphology during pupal development (Figure 3A and B), supporting the idea that gene expression is modulated within changes in the olfactory system itself.

Olfaction is essential for *C. hominivorax* adult life (Devaney et al. 1970; Thomas 1993; Hall 1995). Therefore, it is not surprising that *ChomOrco* is broadly expressed in Screwworm olfactory appendages, mainly in the antennae (Figure 3D and 4G), as expected for its morphology (Figure 3E). Some authors separate the attraction of blowflies to their hosts in three scalable steps, from initial activation to orientation, and landing, culminating in the final decision of egg-laying by gravid females (Ashworth and Wall 1994). Activated olfactory receptors in the Screwworm antennae are likely to evoke flight orientation towards distance attractive host trauma volatile compounds. Indeed, olfactory cues will be the only stimuli detectable by *C. hominivorax* at sites beyond their visual range. In near distances, visual cues encourage the selection of landing sites, and other chemosensory stimuli, as tactile, gustatory and thermal, are though to come into play when the fly reaches the putative host (Hall 1995). These many sensory signals contribute to generating enough stimuli for the egg-laying decision. Interesting, although Orco is mainly related to olfaction, *ChomOrco* is also detected in other non-classical olfactory tissues, including legs and abdomen of both sex, and females' ovipositor (Figure 3D). These observations imply that Orco has a broader chemosensory role in Screwworm - as first suggested from *Drosophila* studies (Vosshall et al. 2000) - and/or those tissues harbor olfactory structures.

Expression of Orco in insect's legs appears to be a conserved characteristic, from locust (Yang et al. 2012) to fig wasps (Lu et al. 2009), mosquitoes (Pitts et al. 2004; Xia and Zwiebel 2006) and true flies (Wang et al. 2012; Olafson 2013). Orco transcription in Screwworm legs indicates a role in contact and close-range chemosensory perception, which might be particularly important for the modulation of dose-dependent response to specific compounds. Putative chemosensory structures present in females' abdominal segments would assist in the detection of host-

associated volatiles, perhaps after landing, and thus enhancing the acquisition of stimuli for oviposition. Abdominal olfactory sensilla and globular olfactory pegs have been described for many Calliphoridae and other related species (Wallis 1962; Ngern-Klun et al. 2007; Chaiwong et al. 2008; Sollai et al. 2010; Olafson 2013). In particular, the study by Sollai et al. (2010) demonstrated that multiporous sensilla on female's abdominal segments of the biting midge, *Culicoides imicola*, are sensitive to host-related compounds, suggesting a role in oviposition site selection, and possible in host localization. More recently, Yadav and Borges (2017) demonstrated that the ovipositor of parasitic fig wasps is, in fact, an olfactory organ, responding to volatile compounds and CO_2. The authors further suggested that other oviparous insects might collect ecologically relevant cues with their ovipositor, building-up sufficient concentration-dependent stimuli for oviposition. Interesting, gravid females of *C. hominivorax* invest a substantial amount of time examining the oviposition sites by walking around while touching the substrate several times with their labellum and an expanded ovipositor. Indeed, this examination process was found to be crucial for egg-laying decision (Devaney et al. 1970). We also observed that Orco mutants spend considerable more time doing this procedure in comparison with wt flies (data not shown), presumably to compensate for their absence of olfaction. Overall, expression data support the idea that olfactory cues are mainly recognized in *C. hominivorax* antennae, and that female's legs, abdomen and ovipositor presumable contribute to the acquisition of short-range and contact stimuli. In this scenario, multiple sensory stimuli seem to be required to trigger egg-laying behavior in Screwworm, but olfaction represents the key to host-seeking behavior.

Investigations carried out over the last half-century have made important progress in understanding the signals that mediate attractiveness of Screwworm to susceptive hosts (see review by Tomberlin et al. (2017) and within references). Early studies demonstrated that gravid females are highly attracted to bacteria-produced volatile organic compounds (VOCs) present in contaminated wounds, mainly with *Providencia* and *Proteus* species. Fresh blood stimulates oviposition on contact, but it seems to be non-attractive to *C. hominivorax* olfaction, although this has been observed in the wild (J. Welch, personal communication). A hypothetical scenario would be; first incursions of Screwworms occur in response to contaminated blood in

fresh wounds. Larvae penetrate and feed on living flesh generating a favored environment for bacteria colonization. The increased production of bacteria-associated VOCs acts as a long-distance signal that attracts other females to lay their eggs, leading to traumatic infestations. Therefore, Screwworm-infested wounds are considerate the favored oviposition sites in nature. However, almost none progress has been made on how those signals are actually perceived by *C. hominivorax*. In a very clever set of experiments, Devaney et al. (1970) first demonstrated that olfactory receptors responsible for host-seeking and oviposition sites selection by *C. hominivorax* are mainly located in the antennae. This was further corroborated by Fernandes et al. (2004), who characterized the olfactory sensilla in the Screwworm antennae. Hammack (1991) also found evidence that contact with host fluids is crucial for recognition and selection of suitable oviposition sites, although olfaction plays a major whole in host-seeking behavior.

In this study, we present another step in understanding the link between the genome and ecological niche occupation by *C. hominivorax*. We used CRISPR genome editing to disrupt *ChomOrco* (Figure 4), as it represents a master gene for insect OR-mediated olfaction (see above). Screwworm Orco mutants are protein null (Figure 4G, and S7) providing means to rapidly expand our knowledge about chemosensory pathways that interplays signal and perception in this species. Screwworm Orco mutants display several development debilities, contrasting the results obtained for *Drosophila* (Asahina et al. 2008) and *Aedes* (DeGennaro et al. 2013). However, fitness reduction was also observed for the Australian sheep blowfly, *Lucilia cuprina* (T. Perry, personal communication) and a number of other insects when Orco gene is disrupted (Koutroumpa et al. 2016; Trible et al. 2017; Yan et al. 2018) or silenced (Franco et al. 2016). Therefore, to preserve the mutant strain while screening the attractiveness of flies, we develop a non-lethal dual-choice trap bioassay (Figure 5). Two-choice trap assays have been widely used in other models to measure the contribution of the olfactory system to specific odor sources (Larsson et al. 2004; Knaden et al. 2012; DeGennaro et al. 2013; Karageorgi et al. 2017). Overall, we found that members of the OR family are indispensable for foraging and host-seeking behaviors in *C. hominivorax* (Figure 5E and G). While wildtype and heterozygous flies are highly attracted to both honey and oviposition media odors, homozygous mutants did not respond to either

odor source, exhibiting a lack of decision-making and disrupted flight-orientation. Experiments with ablated Screwworms revealed that other olfactory organs do not compensate for the absence of antennae to elicit flight-orientation towards odor source. Nevertheless, mutant flies feed and oviposit in rearing conditions, corroborating to previous observations that other sensory systems are involved in oviposition sites selection and egg-laying behavior.

Our behavioral data corroborates with the hypothesis that changes in olfactory preferences, in special mediated by the OR pathway, have contributed significantly to niche shift in the *C*. *hominivorax* lineage. These have important implications for evolutionary studies in blowflies. For instance, there is only another obligatory ectoparasitic screwworm in the world in addition to *C*. *hominivorax*; the Old World Screwworm fly *Chrysomya bezziana*. The Old World genus *Chrysomya* and the New World genus *Cochliomyia* share a common last ancestor at the basis of Chrysomyinae subfamily, spanning roughly 17 Ma of independent evolution (Junqueira et al. 2016). While the Americas consisted in the original territory of *C*. *hominivorax* (now restricted to South America and some Caribbean regions), the natural range of *C*. *bezziana* spans through tropical and subtropical regions of Africa and Asia, where it seems to occur as two geographic races (Hall et al. 2001), and with potential invasion into Australia. These species are separated in time and space, however occupying an exactly equivalent parasitic niche in their natural habitat. Therefore, it would be interesting to investigate if the ORs are also required for host-seeking behavior by *C*. *bezzina*. Another interesting perspective would be to examine the olfactory genes of facultative species of *Lucilia* genus (mentioned before), as their parasitic habit seems to have arisen more recently in response to opportunities created by animal domestication (Erzinclioglu 1989; Stevens and Wallman 2006). These investigations would reveal whether evolution works on the same genomic regions to shaped adaptation in these parasitic blowflies' lineages, extending this convergent evolutionary process from behavior to genetics.

We are currently identifying the chemosensory repertoires of the Screwworm transcriptome in different physiological conditions, focusing on the ORs (R.M. Gonçalves, unpublished). The long-standing goal is to find loci linked to the adaptive evolution of the *C*. *hominivorax*. Candidate genes can be functionally examined using

the CRISPR methods already described for this species (Paulo et al. 2019; and this work). In the future, knock-ins of fluorescent or phenotype markers would be preferred, facilitating mutant screening and strains development. Better fitness is expected for flies carrying mutations in a specific OR, in comparison to Orco knockouts generated here, allowing the maintenance of several mutant strains for different genes. Electroantennogram recordings (EAGs) would be required to confirm the link between genes and chemicals. In an applied point-of-view, this investigation might assist in the identification of species-specific, environmentally safe, and affordable chemicals to be used in the field (e.g., push-pull strategies). That would be not only interesting for countries were Screwworm infestations remain a serious problem, but also as a complement to the current SIT program and future genetic-based control strategies. With the advance of functional genomic tools in *C*. *hominivorax* and other blowflies, it will be possible to genetically manipulate the behavior of parasitic species, for instance changing their host preference, as already possible for *Drosophila* (Matsuo et al. 2007). Therefore, we believe that the data described here will be a landmark for new functional ecology studies in blowflies, encouraging other investigations on the molecular complexity of host preference and speciation in the Calliphoridae family.

With all the respect to the simplistic Zumpt's statement, certainly outstanding for his time, the evolutionary steps that were required for the emergence and evolution of the obligatory ectoparasitic lifestyle in blowflies revealed to be more complicated than he anticipated. We have just started digging into the genome architecture of these species, functionally searching for insights into the genetic basis of such impressive ecological adaptation: From a free-living to an obligatory parasitic fly.

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SUPPLEMENTAL MATERIAL

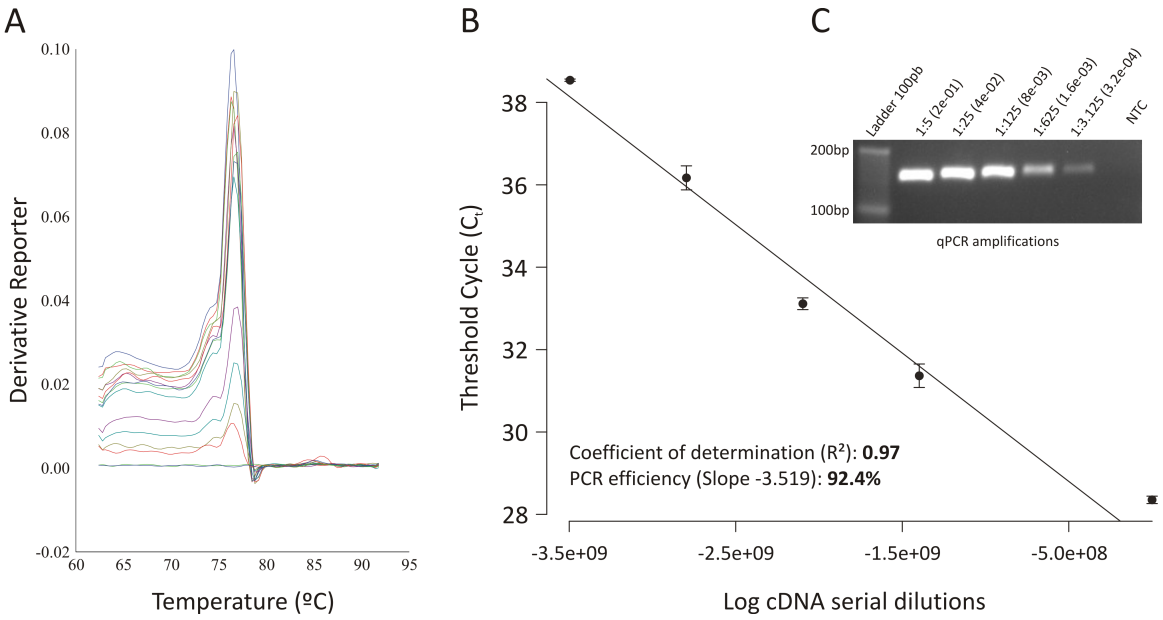

Figure S1 Quantitative PCR (qPCR) efficiency analysis. The amplification efficiency of the Orco-F2 and Orco-R2 primers (Table S1) for qPCR experiments was evaluated by Standard Curve analysis using 5 serial dilutions of cDNA. **(A)** Dissociation curve indicating single specific amplification of *ChomOrco* transcript. **(B)** The Threshold Cycle (C_T) results are presented with the respective standard deviations of the means (n = 3). Efficiency was calculated as: $E = -1 + 10^{-1/slope}$. All amplifications were resolved in a 2% agarose gel electrophoresis, confirming results obtained by dissociation curve analysis.

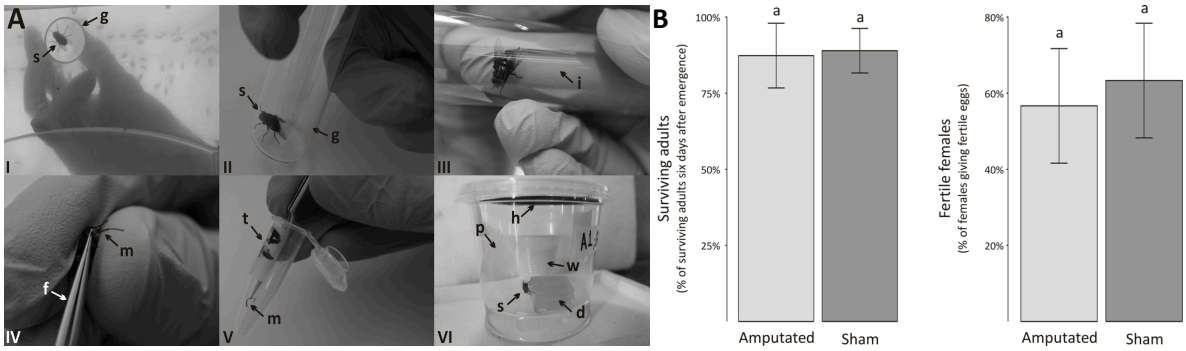

Figure S2 Non-lethal tissue sampling for DNA isolation and genotyping. (A) Upon emergence, adult flies were individually captured in a 30 mL glass vial (25 mm opening Ø, panel I and II). The captured flies were gently held and turned upside down to expose their legs (panel III and IV). A single midleg was then sectioned at the proximal coxa using a forceps (panel IV). Legs were transferred to a 1.5 mL centrifuge tube and maintained at -80° until DNA extractions (panel V), while adults were transferred to an individual plastic cage provided with food and water (panel VI), until molecular genotyping results. **(B)** To evaluate the amputation effects on survival and fertility, dissected wt adult flies were transferred from the individual cages to mating assay cages (three days after leg sampling). Test cages (*n* = 6) were founded with ten amputated individuals each (males and females, ratio 1:1), while sham individuals (e.g., flies that passed through the same sampling procedure but dissection) were used in control cages (*n* = 6). Survival rates were evaluated six days after adult emergence, and females were individually stimulated to oviposit inside a 30 mL glass vial. Viable offspring production was checked on the next day. Bars marked with same letters are not significantly different as given by two-tailed Student's t-test. Results showed that the non-lethal tissue sampling have little or none hazardous effects on screwworm survival and fertility. Abbreviations used: Ld (Ladder 1Kb plus molecular marker); NTC (non-template control); [On Fig S2A] s (Screwworm fly); g (glass vial); i (index finger); f (forceps); m (midleg); t (tube); d (diet); w (water); p (gauze perch); h (holes). We used our custom method for DNA extractions. Dissected legs were physically homogenized in 50 µl of Proteinase K solution (PKS, final concentrations: 0.5 mg/mL Proteinase K, 10 mM Tris-HCL, 1 mM EDTA, and 25 mM NaCL in ddH 2 O), fresh prepared. Mixtures were centrifuged, transferred to 0.2 mL PCR tubes, and incubated at 37° for 60 min followed by enzyme inactivation at 95° for 10 min. Extractions were kept at -20° until PCR assays. Photos credit to Stephanie Mladinich.

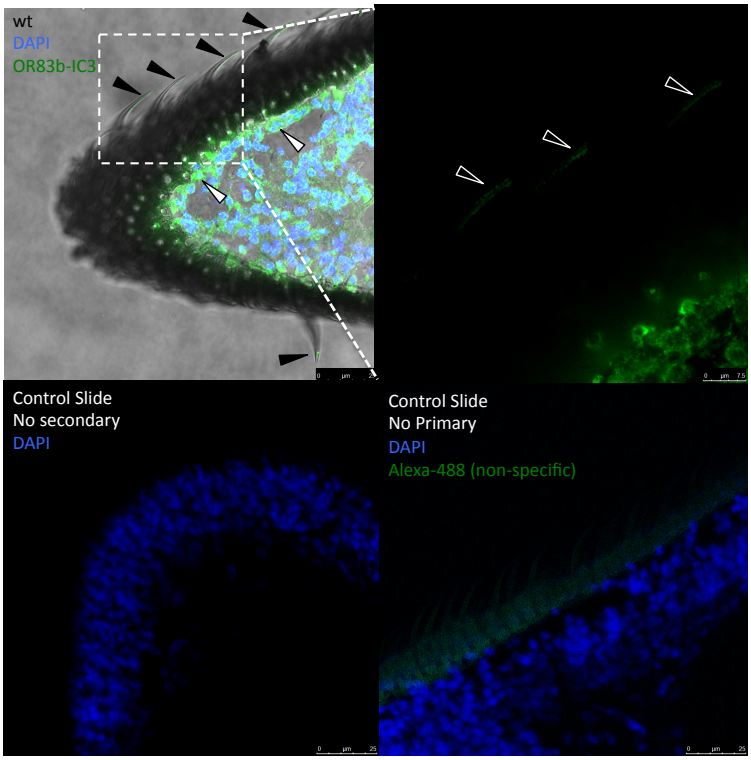

Figure S3 Control slides for immunostaining of *ChomOrco*. Wildtype (wt; above) samples shows that Orco is highly expressed in the cell body (white arrows) and dendrites (inside antennae sensilla; black arrows) of olfactory sensory neurons (OSNs). Control slides (below) showed no specific labeling, but some nonspecific background in the absence of primary OR83b-IC3 antibody (same gain/exposition settings used for wt sample above and samples showed in Figure 5G).

Figure S4 Control trials for two-choice trap bioassays. Response of wildtype flies (*ChomOrco*^{wt}) to honey (honey jar) or glycerol (drop) in two-choice trap bioassays. Adult Screwworms distribute evenly between traps when both contain honey (Student's *t*-test, $P = 0.42$, $n = 11$), and show little or none response to glycerol ($n = 9$). However, flies show a strong preference for honey in opposition to glycerol ($P < 0.001$, $n = 12$). Preference Index was calculated as: PI = $(n_{trap}/n_{total})*100$, where n_{trap} is the number of flies captured in a given trap, and n_{total} the total of flies in the test cage.

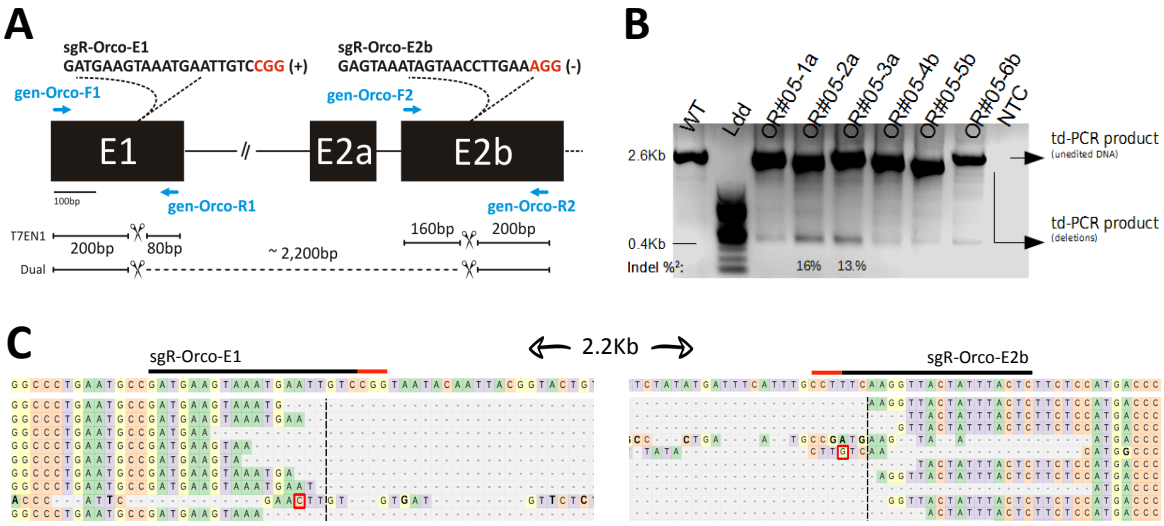

Figure S5 CRISPR/Cas9 dual-targeting approach. In addition to the PCR-based genotyping methods used, we evaluated the possibility of inducing large deletion events between Cas9 targeted regions by injecting RNPs multiplexed with both sgRNAs into embryos. **(A)** The goal was to introduce a ~2.2 Kb deletion at the *ChomOrco* loci between sgR-Orco-E1 and sgR-Orco-E2b targets (gene organization as described in Figure 4A). **(B)** Large deletions are easily distinguished from the wildtype (wt) allele by routine PCR (in this case a Touchdown PCR; td-PCR), followed by electrophoresis. A degree of deletion events was observed from individuals developing from dual-targeting microinjections, but with low efficiency. Complete ~2.2 kb deletion events were rarely seen, accounting for only ~14% of all induced mutagenesis events (estimated using ImageJ software). None large deletions were observed after crossing dual-targeting survivors with wt flies (data not shown). Supposedly, these results reflect the unbalanced efficiency of the designed sgRNAs against ChomOrco (see Figure 4). **(C)** Sequencing confirmation of target-specific deletions between exons E1 and E2b of *ChomOrco*.

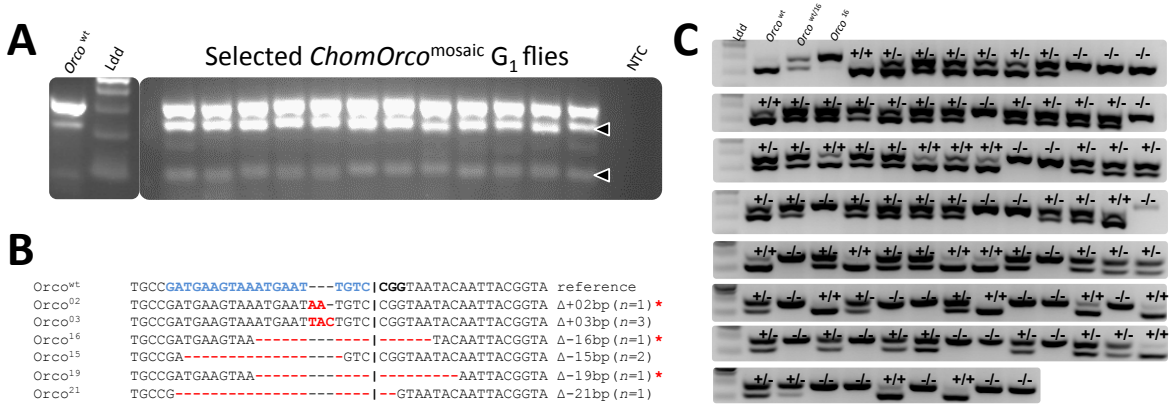

Figure S6 Summary of molecular genotyping results. (A) Genotyping by T7EN1 revealed that all 12 surviving males obtained from microinjections (G_0) hosted indels at the *ChomOrco* exon 1. Ten males were randomly selected and individually backcrossed to virgin wildtype (wt) females to exam their founder capacities. **(B)** Cas9-induced mutated alleles in *ChomOrco* obtained at G_1. The sgR-Orco-E1 sequence is highlighted in blue letters (in the context of wt reference sequence), the PAM motif in black, and the putative Cas9 cleavage site in vertical dashes. The sequence modifications (Δ) are highlighted in red letters (insertions) and in red dashes (deletions). The number of individuals carrying the specified allele is given in parenthesis (*n* = x). Mutated alleles producing premature stop codons within the *ChomOrco* coding sequence are designated with red asterisks (*). **(C)** Siblings carrying a -16 bp deletion at *ChomOrco* ($Orco^{16/wt}$) were selected and inbreed. Their offspring (G_3) were genotyped by Cas9-cleavage assay method, and homozygous mutant flies let to inbreed to establish the mutant strain used in this study. The crossing scheme used to develop the $ChomOrco^{16}$ strain can be found in Figure 4E.

ChomOrco^{wt}

ChomOrco^{16}

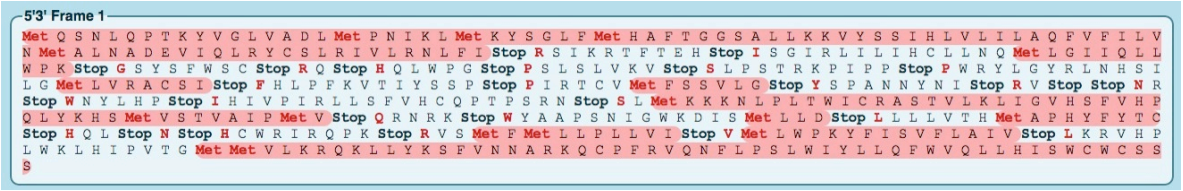

Figure S7 Consequence of the -16bp deletion on *ChomOrco*. Among the developed lines, the one harboring a -16bp deletion ($ChomOrco^{16}$) was selected and inbred to establish a homozygous mutant strain. This knockout mutation was chosen based on the likelihood of functional consequences on *ChomOrco*, as it introduces several premature stop codons on the transcript preventing the translation of a wildtype functional protein ($ChomOrco^{wt}$). Coding sequences were translated using the ExPASy translate tool (Gattiker *et al*., 2003)

Table S1 Primers used in this study.

Name	Target	Aim	Direction	Sequence (5' - 3')	Length	TM	Amplicon	Potential off-targets [d]	mitSpecScore [d]
genOrco-F1	*ChomOrco* exon 1 [a]	genotyping and long-PCR	+	GGT TAT GTC ATG TCG TTA CAG AAG	24 nt	57°	280bp	-	-
genOrco-R1		genotyping	-	GTT CTT TTG ATT GAC CGC TAG AT	23 nt	57°		-	-
delOrco-F1			+	ACT TGT CAA CAT GGC CCT GAA	21 nt	60°	120bp (with R1)		
genOrco-F2	*ChomOrco* exon 2b [a]	genotyping	+	GGA TAA CCA TCA CTT TCT TTG	21 nt	52°	360bp	-	-
genOrco-R2		genotyping and long-PCR	-	TGG CTG ACA ATG AAC GAA AG	20 nt	56°		-	-
Orco-F1	*ChomOrco* exon E2b (3'-end)	RACE and long-PCR	+	GTT CTT GGT TAA TAT TCG CCT G	22 nt	55°	660bp	-	-
Orco-R1	*ChomOrco* exon E5		-	GGC ATT GTT GAC AAA CGA TTT G	22 nt	57°		-	-
Orco-F2	*ChomOrco* exon E2b (3'-end)	qPCR and long-PCR	+	CAA CTA CAA CAT TTG AAG GG	20 nt	52°	174bp	-	-
Orco-R2	*ChomOrco* exon E3 (5'-end)	qPCR and RACE	-	AGA TGC CCG ACA TAT CCA GG	20 nt	59°		-	-
Orco-R3	*ChomOrco* exon E4	long-PCR	-	TTT GGT TGC CTG ATA CGC CA	20 nt	60°	-	-	-
gapdh-F	*ChomGapdh*	qPCR and RT-PCR [b]	+	GTC AGT GAC ACC CAC TCC TC	20 nt	59°	128bp	-	-
gapdh-R			-	TTG ATC AAG TCG ATG ACA CG	20 nt	55°		-	-
sgR-Orco-E1	*ChomOrco* exon 1	sgRNA [c]	+	GAT GAA GTA AAT GAA TTG TC **CGG**	20 nt	-	-	0-0-0-3-27 [e]	93
sgR-Orco-E2b	*ChomOrco* exon 2b		-	GAG TAA ATA GTA ACC TTG AA **AGG**	20 nt	-	-	0-0-0-1-15 [e]	71

[a] Same genotyping primers were also used for Illumina library preparation by including Multiplexing Read 1 (5' – CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT CT – 3') and Read 2 (5' – GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T – 3') Sequencing Primers at the 5'-end of the forward and reverse genotyping primers, respectively;

[b] Primers described by Cardoso *et al*., (2014);

[c] Single guide RNAs (sgRNAs) used in this study, PAM motif in red. Syntheses were performed as described by Bassett and Liu (2014), using the primers sgR- Specific-T7-FWD (5'- GAA ATT AAT ACG ACT CAC TAT A(G) [***specific sgRNA sequence without PAM***] TTG GGT TTT AGA GCT AGA AAT AGC -3') and sgR-Universal-REV (5'- AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATT TCT AGC TCT AAA AC -3');

[d] The sgRNAs were designed by using the standalone version of CRISPOR tool (Concordet and Haeussler 2018). Potential off-targets (see alignments bellow) were evaluated in the context of *C. hominivorax* draft assembly (M.J. Scott, unpublished). We only considered sgRNAs that have potential off-targets with ≥ 3 mismatches in total, with the maximum amount of mismatches present on the 5 – 10 first bases of the sgRNA directly upstream to PAM motif, which constitute the so called "*seed*" region (Andersson *et al*., 2015; Zheng *et al*., 2016);

[e] (Bellow) Alignment of sgRNAs and their potential off-targets predicted by CRISPOR software. Seed region is marked as italic letters, mismatches in red. Only off-targets with ≤ 3 mismatches are shown.

```
sgR-Orco-E1   GATGAAGTAAATGAATTGTC   sgR-Orco-E2b GAGTAAATAGTAACCTTGAA
off-target-1  GAaGAAGTAAATGtATTGTt   off-target-1 GAGTAAATAGgAACCTTtgA
off-target-2  GATcAAGaAAcTGAATTGTC
off-target-3  GATGAAtTAAATGAATTaaC
```

Table S2 Evolutionary sequence divergence of Orco genes between dipteran species. Uncorrected evolutionary distances (*p*-distance) based on nucleotide (nt), and amino acid (aa) sequences were estimated using pairwise comparisons with pairwise site-deletions in MEGA7 (Kummar et al. 2016). Results are shown as Avg ± SE.

Sequence	Comparison	*p*-distance
nt	*ChomOrco* vs Diptera	0.21 ± 0.006
	ChomOrco vs Calyptratae	0.13 ± 0.004
	ChomOrco vs Calliphoridae	0.08 ± 0.004
	ChomOrco vs *DmelOrco*	0.27 ± 0.010
	ChomOrco vs *CmegOrco*	0.08 ± 0.007
aa	*ChomOrco* vs Diptera	0.08 ± 0.006
	ChomOrco vs Calyptratae	0.03 ± 0.004
	ChomOrco vs Calliphoridae	0.02 ± 0.005
	ChomOrco vs *DmelOrco*	0.09 ± 0.013
	ChomOrco vs *CmegOrco*	0.02 ± 0.005

Table S3 Mutational inheritance of Orco mutant alleles. Injected Screwworm males (putative founders) were genotyped using our non-lethal DNA extraction method (Figure S2) and T7EN1 cleavage assay (Figure 4). Ten confirmed heterozygous flies ($Orco^{wt/-}$) were randomly selected and individually backcrossed to wt Screwworm females. For each crossing, another 8 males (on average) were randomly sampled and genotyped as before. Transmission efficiency was found to be 90% (e.g., 9 out of 10 crosses produced heterozygous offspring), while inheritance success(e.g., germline transmission) ranged from 14.3% to 88.9%. In total, 41% (*n* = 38/81) of the genotyped flies were found to be heterozygous.

Crossing ID	$Orco^{wt/-}$	$n\ G_1$	Inheritance
A	3	8	37,5
B	8	9	88,9
D	2	14	14,3
E	1	6	16,7
G	3	4	75,0
H	5	8	62,5
L	2	6	33,3
N	4	5	80,0
O	0	8	0,0
Q	10	13	76,9

THE END

General Conclusions

CHAPTER 1

- In Chapter 1 we described for the first time the successful implementation of CRISPR/Cas9 site-directed mutagenesis methods for *in vivo* functional analysis of candidate genes in the New World Screwworm fly, *Cochliomyia hominivorax*.

- CRISPR/Cas9 loss-of-function experiments genetically confirmed the orthology of the *C. hominivorax yellow* gene (*ChY*). As hypothesized, mutations at this gene resulted in the non-lethal *brown body* (*bwb*) phenotype, and thus could be used to detect and score Cas9-induced mutagenesis in Screwworm.

- Using the *ChY* as a marker, we developed high efficient protocols for the delivery of pre-assembled Cas9:sgRNAs complexes into nearly laid Screwworm embryos. Our results showed that these protocols are likely to induce high rates (~68%) of somatic biallelic lesions at G_0.

- Most of the mutated cells were visible around the injection site. Since embryos were injected at the posterior end it was expected that mutations would be found in the germline, and thus would be inheritable. That was further confirmed by crossings between mosaic *bwb*-mutant flies. Due to the inheritance nature of Cas9-induced mutations, we were able to develop the *ChYellow*$^{07/01}$ strain, demonstrating the potential of CRISPR/Cas9 in reverse genetic studies of *C. hominivorax*.

- Low concentrations of Cas9 (360 ng/µl) was found to increase survival of injected embryos to adults (from 9% to 17%), but lower mutagenesis efficiency at G_0 (74% to 56%) as well as inheritance rates (from 82% to 38%, in average) when compared to the high Cas9 concentration tested (500 ng/µl). Therefore, we suggest that for most targets a midrange concentration (around 400 ng/µl) would be suitable to balance survival and mutagenesis efficiency, although high concentrations might be desired when studying recessive autosomal markers in order to favor biallelic hits.

- Several colonies of the *ChYellow*$^{07/01}$ mutant strain has been maintained at COPEG rearing facility for over 21 generations (last checked December 2019), demonstrating that mutations introduced by Cas9:sgRNA RNPs are stable. The *ChYellow*$^{07/01}$ strain might be a useful recipient for future germline transformation (e.g., Knock-in experiments) with vectors containing *ChY*$^{+}$ as the marker gene. Transformed flies would be easily identified through the rescue of the wt body color. This would be particularly interesting when studying non-visual gene traits.

- Knockout experiments against the *transformer* gene (*Chtra*) indicates that the Cas9-mediated mutagenesis protocols developed in the present study are suitable for inducing mutations in other regions of the Screwworm genome.

- Cas9-induced mutations in the *Chtra* gene led to the masculinization of XX flies, demonstrating that this gene is required for normal female development in *C. hominivorax*, as previously showed in other closely related species, and revealing its evolutionary conservation among blowflies.

- The *Chtra* should be considerate a target for "*homing*" CRISPR gene-drive systems, which could be an effective means for producing only males (e.g., sexing-strains) for a more efficient SIT program. The efficiency of this strategy on the field remains to be better evaluated.

- While 60% of surviving females from knockout experiments against *Chtra* developed an intersex phenotype (i.e., individuals carrying male and female characteristics simultaneously) with different degrees of masculinization, we were unable to identify potential fully transformed XX flies due to the current lack of sex-linked genetic markers for *C. hominivorax*. Future functional genomic investigations should be able to identify genes for sex-linked traits in Screwworm. A potential candidate would be the *black* (*Chbl.*) locus. The *Chbl* loss-of-function is supposed to result in a body-color change from the normal blue-green to jet black in Screwworm[5], allowing the identification of XX and XY flies in future examinations on sex determination in this species.

- While the implementation of novel genetic-based methods aiming the Screwworm suppression in the wild remains to be evaluated, the availability of CRISPR knockout protocols described here will allow us to functionally interrogate the genetic bases of *C. hominivorax* physiology, development and evolution.

C H A P T E R 2

- In Chapter 2 we isolated, characterized, and functionally confirmed the orthology of the Odorant Receptor Co-receptor (Orco) gene of *Cochliomyia hominivorax* (*ChomOrco*).

- The *ChomOrco* gene is highly conserved, sharing an extremely high amino acid identity with all dipterans investigated in this study (mean ± SD: 92 ± 5.5%). In addition to its sequence, the genomic organization of *ChomOrco* is also conserved among schizophoran flies, including the presence of the presumable Calyptratae' specific intron I1b.

- Tests for site-specific signatures of episodic diversification revealed that *ChomOrco* conservation is maintained due a strong purifying selection regime in the majority of the corresponding amino acid sites (97.3%), while remain sites

[5] LaChance, L.E. and Hopkins, D.E. (1962). Mutations in the Screw-Worm Fly. *Journal of Economic Entomology* 55(5): 733–737.

showed evidence of relaxed constraint. Similar evolutionary signatures were obtained for other lineages, revealing Orco indispensable role across taxa.

- *ChomOrco* expression profiles during development indicate that the Screwworm olfactory system experience drastic changes between immature (larvae) to adult stages. Detection of Orco in early larvae might indicate the usage of olfactory clues to find suitable substrate to feed, while low expression of Orco during the last larvae stage might be related to the "*host exodus*" behavior. The *ChomOrco* expression is correlated to changes in the peripheral olfactory morphology during pupae development, resulting in its higher expression in the adult stage, when olfactory appendages are fully developed. The same patterns were found during the development of the blowfly *Chrysomya megacephala*, indicating that regulatory mechanisms of Orco expression are evolutionarily conserved as well.

- *ChomOrco* is broadly detected in the olfactory tissues of *C*. *hominivorax* adult stage (e.g., antennae and maxillary-palps), in agreement with their morphology. *ChomOrco* expression in the antennae was found to be critical for initial activation evoking flight orientation towards distance host-associated volatile compounds (see below). The presence of *ChomOrco* expression in other non-classic olfactory appendages (e.g., legs, abdomen, and ovipositor) might contribute to generate sufficient stimuli (in a close-range or by contact) for egg laying decision during the "*examination walking*" behavior. While additional morphological experiments are required to verify the presence of olfactory structures in these appendages, *ChomOrco* might also have a broader chemosensory role in Screwworm.

- CRISPR dual-targeting approach seems to be a promising method, not only for PCR-based genotyping strategies (in addition to the ones described here) but also for the examination of large DNA motifs on the genome. Regarding genotyping, knock-ins of fluorescent or phenotype markers would be preferred in the future, which would facilitate mutant strains development. Both strategies remain to be better evaluated in the Screwworm system.

- Homozygous Screwworm Orco mutants display a lack of decision-making and disrupted flight-orientation towards both honey and oviposition-media odors, suggesting that the Odorant Receptor (OR) mediated olfactory pathway is indispensable for foraging and host-seeking behaviors in *C. hominivorax*.

- Behavioral bioassays also indicate that evolution might be acting on the OR gene family members, rather than on key genes such as Orco, to architect the olfactory landscape related to host-seeking behaviors of the Screwworm fly. Therefore, it would be interesting to investigate whether the same gene family is related to the obligatory ectoparasitic habit of the Old World Screwworm fly, *Chrysomya bezziana*, and to the primary facultative habit of *Lucilia* species.

- An obviously (and underway) next step would be the identification of the OR repertoire in the genome of *C. hominivorax*. The ORs genes can be interrogated for signals of episodic diversification and differential expression in contrasting physiological conditions (virgin x mated). Candidate genes can be functionally examined using CRISPR methods described in this study. Better fitness is expected for flies carrying mutations in a specific OR, allowing the maintenance of several mutant strains for different genes.

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APPENDIX A[6]

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SCIENTIFIC REPORTS

OPEN

Large-scale mitogenomics enables insights into Schizophora (Diptera) radiation and population diversity

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True flies are insects of the order Diptera and encompass one of the most diverse groups of animals on Earth. Within dipterans, Schizophora represents a recent radiation of insects that was used as a model to develop a pipeline for generating complete mitogenomes using various sequencing platforms and strategies. 91 mitogenomes from 32 different species were sequenced and assembled with high fidelity, using amplicon, whole genome shotgun or single molecule sequencing approaches. Based on the novel mitogenomes, we estimate the origin of Schizophora within the Cretaceous-Paleogene (K-Pg) boundary, about 68.3 Ma. Detailed analyses of the blowfly family (Calliphoridae) place its origin at 22 Ma, concomitant with the radiation of grazing mammals. The emergence of ectoparasitism within calliphorids was dated 6.95 Ma for the screwworm fly and 2.3 Ma for the Australian sheep blowfly. Varying population histories were observed for the blowfly *Chrysomya megacephala* and the housefly *Musca domestica* samples in our dataset. Whereas blowflies (n = 50) appear to have undergone selective sweeps and/or severe bottlenecks in the New World, houseflies (n = 14) display variation among populations from different zoogeographical zones and low levels of gene flow. The reported high-throughput mitogenomics approach for insects enables new insights into schizophoran diversity and population history of flies.

True flies are insects that undergo complete metamorphosis and belong to the two-winged insects order Diptera. They represent one of the most diverse groups in the Kingdom Animalia[1] and are structured into two major sub-orders: Lower Diptera (mosquitoes) and Brachycera (short-horned flies). Worldwide, more than 150,000 species have been described[2], making them one of the most successful groups on Earth that occupy almost every terrestrial niche. They parasitize plants and animals, act as biological and mechanical vectors of diseases, serve as biological control agents, as well as model organisms for science[3]. The clade Schizophora contains the majority of the family level diversity[3,4] among Dipterans and represents a recent rapid radiation of lineages. The resulting relationships among Schizophora families remain a challenge for fly phylogeny[2,3]. The rapid radiation in combination with a low extinction rate has led to a diversity that surpasses even the number of all terrestrial species of vertebrates[2–4]. The scarcity of fossil records and the sparse availability of genetic data make Schizophora an interesting target for large-scale molecular evolutionary analyses. These analyses were historically based on mitochondrial markers consisting of single genes or short sequence intervals. More recently, complete mitochondrial genomes have become commonly used for in-depth phylogenetic and population studies[5]. In particular for vertebrates, generation of complete mitochondrial genomes is well established[6], including those of extinct species[7–10]. Sequencing of complete mitochondrial genomes has been proven to refine resolution of relationships both between and within species[11–16], in addition to improving the characterization of genome evolution[17,18] and patterns of substitution rate[19].

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[6] Junqueira et al. (2016), on the evolution of blowflies (Diptera: Calliphoridae).
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APPENDIX B[7]

INVESTIGATION

Specific Gene Disruption in the Major Livestock Pests *Cochliomyia hominivorax* and *Lucilia cuprina* Using CRISPR/Cas9

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ABSTRACT *Cochliomyia hominivorax* and *Lucilia cuprina* are major pests of livestock. Their larvae infest warm-blooded vertebrates and feed on host's tissues, resulting in severe industry losses. As they are serious pests, considerable effort has been made to develop genomic resources and functional tools aiming to improve their management and control. Here, we report a significant addition to the pool of genome manipulation tools through the establishment of efficient CRISPR/Cas9 protocols for the generation of directed and inheritable modifications in the genome of these flies. Site-directed mutations were introduced in the *C. hominivorax* and *L. cuprina yellow* genes (*ChY* and *LcY*) producing lightly pigmented adults. High rates of somatic mosaicism were induced when embryos were injected with Cas9 ribonucleoprotein complexes (RNPs) pre-assembled with guide RNAs (sgRNAs) at high concentrations. Adult flies carrying disrupted *yellow* alleles lacked normal pigmentation (*brown body* phenotype) and efficiently transmitted the mutated alleles to the subsequent generation, allowing the rapid creation of homozygous strains for reverse genetics of candidate loci. We next used our established CRISPR protocol to disrupt the *C. hominivorax transformer* gene (*Chtra*). Surviving females carrying mutations in the *Chtra* locus developed mosaic phenotypes of transformed ovipositors with characteristics of male genitalia while exhibiting abnormal reproductive tissues. The CRISPR protocol described here is a significant improvement on the existing toolkit of molecular methods in calliphorids. Our results also suggest that Cas9-based systems targeting *Chtra* and *Lctra* could be an effective means for controlling natural populations of these important pests.

KEYWORDS
Functional genomics
reverse genetics
myiasis
New World Screwworm fly
Australian Sheep Blowfly
gene drive
CRISPR/Cas9
brown body
yellow
transformer

The New World Screwworm fly, *Cochliomyia hominivorax*, is a major livestock pest and the only obligatory ectoparasitic blowfly in the Neotropical region (Alexander 2006). Likewise, the Australian sheep blowfly, *Lucilia cuprina*, is the main species involved in primary flystrike in Australia and New Zealand (Sandeman *et al.* 2014). Adult females of these species are attracted to oviposit by odors emanated by their hosts (Zhu *et al.* 2017; Yan *et al.* 2018). After hatching, their larvae (maggots) infest and feed on host's living flesh to complete development, which can ultimately lead to lethality if untreated. These infestations (known as myiasis) are responsible for severe economic impacts to the livestock industry, estimated in hundreds of millions spent annually in treatment, prevention and animal welfare (Alexander 2006; Grisi *et al.* 2014). Over the last 60-years, the Sterile Insect Technique (SIT) program has been successfully used to eradicate *C. hominivorax* from all North and Central America (see review by Scott *et al.* (2017)). Currently, the mass rearing biosecurity facility, at the Panama-United States Commission for the Eradication and Prevention of Screwworm (COPEG), is responsible for the dispersal of millions of factory-grown radiated-sterile screwworm males and females in the Darien

[7] Paulo et al. (2019), on CRISPR/Cas9 in Screwworm and Blowfly.
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APPENDIX C[8]

Insect Molecular Biology

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Identification and characterization of microRNAs in the screwworm flies *Cochliomyia hominivorax* and *Cochliomyia macellaria* (Diptera: Calliphoridae)

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs that modulate gene expression through post-transcriptional regulation. Here, we report the identification and characterization of miRNAs in two closely related screwworm flies with different feeding habits: *Cochliomyia hominivorax* and *Cochliomyia macellaria*. The New World screwworm, *C. hominivorax*, is an obligatory parasite of warm-blooded vertebrates, whereas the secondary screwworm, *C. macellaria*, is a free-living organism that feeds on decaying organic matter. Here, the small RNA transcriptomes of adults and third-instar larvae of both species were sequenced. A total of 110 evolutionarily conserved miRNAs were identified, and 10 putative precursor miRNAs (pre-miRNAs) were predicted. The relative expression of six selected miRNAs was further investigated, including miRNAs that are related to reproduction and neural processes in other insects. Mature miRNAs were also characterized across an evolutionary time scale, suggesting that the majority of them have been conserved since the emergence of the Arthropoda [540 million years ago (Ma)], Hexapoda (488 Ma) and Brachycera (195 Ma) lineages. This study is the first report of miRNAs for screwworm flies. We also performed a comparative analysis with the hereby predicted miRNAs from the sheep blowfly, *Lucilia cuprina*. The results presented may advance our understanding of parasitic habits within Calliphoridae and assist further functional studies in blowflies.

Keywords: *Cochliomyia hominivorax*, *Cochliomyia macellaria*, screwworm fly, blowfly, myiasis, parasitism, microRNAs.

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Introduction

In the last decade, a large number of small noncoding RNAs (sncRNAs) has been discovered in several species, including nonmodel organisms (Belles *et al.*, 2012; Kozomara & Griffiths-Jones, 2014), mainly as a result of the advent of high-throughput sequencing and new bioinformatics strategies. Amongst them, microRNAs (miRNAs) has received particular attention owing to their critical role in fine-tuning complex biological processes, such as development and metamorphosis (Cristino *et al.*, 2011; Zhang *et al.*, 2012; Wu *et al.*, 2013; Lozano *et al.*, 2015), immune response (Choi & Hyun, 2012; Hussain & Asgari, 2014), phenotypic plasticity and behaviour (Greenberg *et al.*, 2012; Li *et al.*, 2013; Yang *et al.*, 2014). Mature miRNAs are single-stranded, endogenous sncRNAs of approximately 22 nucleotides (nt) in length that modulate gene expression at the post-transcriptional level (Asgari, 2013; Lucas & Raikhel, 2013). In animals, the partial complementarity between miRNAs and target sites in messenger RNAs (mRNAs) leads to transcriptional decay through translational inhibition, transcript degradation or both (Brennecke *et al.*, 2005; Filipowicz *et al.*, 2008; Schnall-Levin *et al.*, 2010; Schirle *et al.*, 2014).

The first miRNA repertoire of an insect species was reported for the fruit fly *Drosophila melanogaster* (Pasquinelli *et al.*, 2000; Lagos-Quintana *et al.*, 2001). Since then, miRNAs have been described for a number of insects, including 22 species of dipterans (until April 2016). The order Diptera (true flies) is traditionally split

[8] Paulo et al. (2017), on microRNAs in Screwworm flies.
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APPENDIX D[9]

Running Title

Characterization of Orco in Screwworm

Title

Molecular and Functional Characterization of the Olfactory Receptor Co-receptor Orco leads to evolutionary insights into the olfactory-mediated behaviors in the New World Screwworm Fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae).

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[9] Paulo et al. (Unpublished), on Olfaction in Screwworm.
Cover page of the manuscript derived from Chapter 2.

APPENDIX E

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CBMEG

CIDADE UNIVERSITÁRIA "ZEFERINO VAZ",
01 DE OUTUBRO DE 2019.

CIBIO: 05/2019

IDENTIFICAÇÃO

Doutorado: Daniel Fernando Paulo – Comissão de Pós-Graduação – Genética e Biologia Molecular – Instituto de Biologia.

PROJETO

From genes to traits: a functional genomic study on the new world screwworm fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae)

PARECER

Projeto aprovado pela CIBio / CBMEG sob número 01/2018 – Caracterização molecular de genes quimiossensoriais e transcriptoma da antena da mosca da bicheira, *Cochliomyia hominivorax* (Diptera: Calliphoridae).

Coordenador: Profa. Dra. Ana Maria Lima de Azeredo-Espin

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APPENDIX F

Declaração

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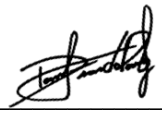
Campinas, 20 de Janeiro de 2020



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